

NUCLEAR PROTEINS DURING GROWTH AND DIFFERENTIATION OF
MOUSE NEUROBLASTOMA CELLS

By

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Mouse neuroblastoma cells in culture can be induced by several methods to "differentiate" into cells which exhibit morphological, biochemical and electrophysiologic properties of neurons. In an attempt to develop a model for the understanding of the regulation of gene expression which must occur during the process of "differentiation," the composition and transcriptional capacity of chromatin and the metabolism of nuclear histones and nonhistones were compared in nuclei isolated from mouse neuroblastoma 1300 cells grown under different conditions. Serum withdrawal or addition of 1 mM dibutyryl cAMP resulted in reversible morphologic "differentiation" in 70 to 90% of the cells, although cell number continued to increase in cultures exposed to dibutyryl cAMP. Chromatin isolated from purified nuclei was transcribed in a cell-free RNA synthesizing system using E. coli RNA polymerase. Chromatin from cells grown in serum exhibited a significant increase in template activity over that observed in chromatin from "differentiated" cells. The synthesis,

acetylation, and phosphorylation of histones and nonhistones were examined by pulse-labelling cells for 60 minutes with appropriate precursors. Chromosomal proteins were analyzed on SDS- and acetic acid-urea polyacrylamide gels. Differences in the synthesis of chromosomal proteins (histones and nonhistones) from cells grown under different conditions were observed. Significant differences were not detectable in acetylation or phosphorylation of the chromosomal polypeptides. A functional role for the observed variations in chromosomal proteins which accompany mouse neuroblastoma cell "differentiation" is discussed.



Chairman

CHAPTER I

INTRODUCTION

The numerous types of cells which exist in the mature multicellular organism are thought to result from the differential expression of information contained in the genetic material of the cells. Each cell in the organism contains at least one complete set of genes, yet the alterations in the morphological and functional properties of the cells are the ultimate product of the expression of only a portion of the genome. The progressive alterations in the biochemical makeup of cells during their development are known collectively as cellular differentiation and are the result of complex interactions of the nucleus, the cytoplasm, and the environment of the individual cell. Mouse neuroblastoma cells in in vitro culture show alterations in morphological and biochemical characteristics which are similar to those described for developing neurons. These cells serve as a useful model to investigate the nuclear events involved in the differential expression of genetic information.

The establishment of the differentiated state is often accompanied by a decreased capacity for cellular proliferation. This relationship, however, varies with respect to its application to different tissues and cell types. In some tissues only limited groups of "stem" cells can divide and replace or add to the number of cells of the tissue. The stem cells themselves are differentiated in the sense that they are specialized to divide and produce a limited number of types of progeny.

The central nervous system of vertebrates, in fact, shows the most complex degree of specialization of cell morphology and function of all eukaryote tissues. The cellular components are all derivatives of the neuroectoderm and its subsequent subdivisions, the neural crest and the neural tube. Yet, each neuron is essentially unique in its position in space and its relationship to other cells, both by direct contact with cells in its immediate environment and via neurohormones with cells in distant regions of the nervous system. Correlated with such specialization is an extreme limitation in the ability of the neuron to recover from injury or to proliferate. How does such specialization of morphology and function result from the developmental processes involved in the maturation of the nervous system? How do cells use the information in their environment and in their genetic material to develop a defined cell shape, specific contacts, and specific products, such as neurotransmitters and neurohormones? Such activities reflect the highly organized utilization of the information contained in the genome of the cell via mechanisms common to all cell types which result in the ultimate synthesis of specific enzymes and structural proteins.

Regulation of Genetic Information

The regulation of the expression of genetic information in eukaryotic cells may occur at several stages in the progression of the transcription of the genes encoded in DNA into RNA, the processing and transport of RNA from the nucleus into the cytoplasm, and the translation of the mRNA into specific proteins in the cytoplasm. Although control may be exerted in mammalian cells at all levels, we will focus on a discussion of regulation of gene expression at the transcriptional level and review

studies on nervous tissue which describe early neuronal differentiation and biochemical studies of nuclear proteins. An in vitro system which has provided much information about cellular and biochemical processes occurring in neuron-like cells will be described and the results of studies of the nuclear proteins of these neuroblastoma cells will be presented.

Regulation of Gene Expression in Eukaryotic Cells

The genetic material in eukaryotic cells, coded in DNA, exists in the interphase nucleus, as seen with the light and electron microscopes, as dispersed chromosomal material--a heterogeneous collection of fibrils and dense material. The more dense regions have been called heterochromatin and are thought to represent tightly coiled, inactive regions of the genome. The less dense regions, known as euchromatin, are thought to represent regions of the DNA that are actively being transcribed. These same terms, heterochromatin and euchromatin, have been applied to various fractions of material composed of nucleic acids and protein which have been extracted by several biochemical methods from cell nuclei. The wide usage of the term "chromatin" to describe such fractions implies that such material, in fact, represents the chromosomes. Actually, it is an operational definition, referring to whatever material has been extracted from the cells. Although the material may reflect the composition of genetic material in vivo, caution should be exercised in interpreting data from studies in which different methods of isolation of chromatin are employed.

Information from numerous studies on biochemically defined chromatin suggests that the proteins associated with the DNA are involved intimately in both the maintenance and establishment of the structure of the genetic

material and in regulating the "readout" or transcription of specific genes. The nuclear proteins fall into two major categories: histones and nonhistones, or acidic proteins. The composition, distribution and suggested role of both classes of proteins have been extensively reviewed recently and only a brief summary will be presented (Stellwagen and Cole, 1969; DeLange and Smith, 1971; Phillips, 1971; Spelsberg *et al.*, 1972b; Elgin and Weintraub, 1975; Stein *et al.*, 1974, Stein *et al.*, 1976 (submitted for publication); Cameron and Jeter, 1974; and MacGillivray and Rickwood, 1974).

Histones

The histones are a defined group of acid-extractable proteins of relatively small molecular weight (about 11,000 to 22,000 for monomeric forms) which are present in the somatic cell nuclei of all multicellular organisms. In general they contain large amounts of basic amino acids (arginine and lysine) and lack tryptophan, and cysteine (found only in H3 histone). They are extremely stable and carry a net positive charge at pH 8. The amino acid sequence and structure of the histone subclasses are amazingly well conserved throughout the plant and animal kingdoms. Each of the five subclasses of histones shows a skewed arrangement of amino acid residues such that the basic amino acids are found in clusters. This asymmetry is apparently involved in the mode of interaction of the specific histones with one another and with other components of the genome, including the DNA and nonhistone proteins.

In addition to slight differences in amino acid sequence, histones are among the most highly modified proteins. Post-translational modifications involve the covalent addition or removal of acetyl, phosphate or methyl groups or the modification of the sulfur group in cysteine.

These post-synthetic modifications of the histone polypeptides have been implicated in structural and functional alterations in the genome. Such modifications would lead to alterations in histone-DNA, histone-histone, and histone-nonhistone interactions.

Involvement of histones in the regulation of gene expression has been suggested since the 1940's. However, the marked conservation of histone structure throughout tissues and species, the lack of tissue specificity with respect to the distribution of histones, and their limited heterogeneity suggest that histones by themselves do not regulate defined gene loci. The major type of evidence linking histones to gene regulation involves their role in the structural organization of the genome and their ability to inhibit DNA-dependent RNA synthesis--i.e., to function as structural and repressor macromolecules. Early studies showed that increasing amounts of histones complexed with DNA resulted in a progressive decrease in DNA-dependent RNA synthesis. In fact, transcription is completely inhibited when the template consists of DNA and histone in a 1:1 ratio (Huang and Bonner, 1962; Allfrey *et al.*, 1963). Consistent with this, histone synthesis in proliferating systems has been found to be tightly coupled to DNA synthesis and occurs only during a restricted period of the cell cycle both in cells in vivo and in vitro.

Nonhistone Proteins

In contrast, the nonhistone proteins represent a far more heterogeneous class of proteins which are synthesized throughout the cell cycle. They do exhibit species and tissue specificity and differ in their distribution and metabolism in the same cell type throughout the cell cycle and functional state of their tissue of origin. These proteins

as a group contain relatively large quantities of acidic amino acids. A few are extractable in dilute mineral acid along with the histones. The class of nonhistone proteins includes polypeptides which function as structural, regulatory and enzymatic macromolecules. The evidence for nonhistone proteins playing a specific role in the regulation of gene activity includes their ability to confer specificity on the transcriptional capacities (quantitative and qualitative) of isolated DNA plus histone material ("reconstituted chromatin"). When chromatin is reconstituted by mixing DNA plus histone plus nonhistone protein fractions, the specificity of the RNA which is recovered from the assay system is dependent on the source of the nonhistone protein fraction. For example, when chromatin is reconstituted from tissue A DNA and histone and tissue B nonhistone fractions, the RNA is similar to RNA transcribed from tissue B chromatin (Gilmour and Paul, 1970; Spelsberg and Hnilica, 1970; Stein and Farber, 1972).

Post-translational modifications of nonhistone proteins have also been implicated in the selective binding of nonhistone chromosomal proteins to homologous DNA.

Several systems are being intensively studied in order to elucidate the role of the chromosomal proteins in the regulation of the readout of specific gene sequences, which include erythropoietic systems and the transcriptional regulation of the globin gene (Paul et al., 1973), HeLa cells and WI38 fibroblasts and the regulation of the histone genes (Stein et al., 1975), and chick oviduct cells and the regulation of the ovalbumin gene (O'Malley and Schrader, 1976).

Early Neuronal Maturation

Most of the large neurons of the central nervous system arise from

the outer region of the wall of the neural tube known as the primary germinal centers (Phelps and Pfeiffer, 1975). The neural tube itself originates as an invagination of the neural ectoderm or neural plate very early in development (Karfunkel, 1974; Burnside and Jacobson, 1968; Schroeder, 1970). Cell shape changes, alterations in cellular adhesion, and regional proliferation have been described as participating in neurulation and neuronal differentiation. The underlying molecular events in such developmental processes involve recognition of changes in the external environment of the cells by the cell membrane and its constituents and the translocation of signals to the nucleus to alter or initiate the production of mRNAs which code for components which can in turn result in changes in cell shape and so on. In order to study such events a relatively homogeneous population of cells is required. One of the greatest barriers to such research in nervous tissue in vivo is the ability to obtain sufficient material to analyze.

We will describe the major early changes in morphology of neuroblasts as they leave the mitotic population of the neural tube and then review briefly what is known about biochemical differentiation. Cowdry (1914) reported on an intensive, light-microscopic study, using several histologic stains, of developing components of the neural tube of the chick embryo. He reported that the first observable alteration in the cytoplasmic constituents of neural tube cells involved the appearance of neurofibrils (which show up after silver impregnation of tissue) in the cytoplasm of cells at the outer margin of the neural tube. These neurofibrils formed first in a definite restricted zone of the cytoplasm on the side of the nucleus away from the lumen of the neural tube. Nissl substance, intensely basophilic material arranged in clumps, appeared

much later. Mitochondria were numerous in the peripheral regions of the cells.

More recent studies utilizing both light and electron microscopy (Bellairs, 1959; Fujita and Fujita, 1963; Eschner and Glees, 1963; Lyser, 1964; Meller et al., 1966; and Decker, 1974) confirm the late appearance of Nissl substance and indicate that it is the light microscopic counterpart of the rough endoplasmic reticulum which becomes organized into arrays of ribosome-studded membranes as differentiation progresses. Early changes in the maturing neuroblasts include the decrease in yolk droplets present in the cytoplasm, increased numbers of mitochondria (especially in elongating regions of the cells), and appearance of feltworks of filaments (60-130 A in diameter). Correlations made between light microscopic, silver-impregnated material and electron microscopic studies, suggest that filaments may correspond to silver-staining material. In fact, silver granulations in the cytoplasm on the side of the nucleus away from the neural tube lumen are the earliest changes which have been reported. Neurofibrils appear later.

Cells develop a long external process whose distalmost end appears identical to growth cones described in slightly older material and in tissue culture studies. As differentiation progresses, the cell becomes monopolar as its internal process is withdrawn. The nucleus becomes more uneven in texture and granular endoplasmic reticulum becomes more extensive. The Golgi material seems to become aggregated and organized around the nucleus. Centrioles may or may not be present. Secondarily, the cells become bipolar as a dendrite-like process which is morphologically indistinguishable from the initial axon-like process develops. Nissl substance appears simultaneously with the development of bi- and

multipolarity. No endings are yet recognizable on either cell bodies or developing processes.

As mentioned earlier, subsequent development varies from region to region. Morest (1969) and Peusner (1974) have described the development of neurons or "post-mitotic neuroblasts" in the brainstem, Hinds (1968a, b) in the olfactory bulb, Rakic (1973) in the cerebellum, and Berry and Rogers (1965), and Butler and Caley (1972) in the cerebral cortex.

Jacobson (1974), in an interesting essay, has suggested that all neurons may be considered to belong to one of two major subpopulations. One of these, usually composed of larger neuron types, seems to exhibit invariant morphology and connectivity, and the other seems to be more flexible in its relationships. He suggests that this dichotomy reflects basic differences in the regulation of genetic information to produce constant phenotypes in the larger neurons and more variable ones in the smaller neurons as a result of the latter cell population's ability to respond more readily to alterations in the cellular environment. This is probably a gross oversimplification of regulatory mechanisms, but perhaps sets up a framework within which to compare regulatory events in neurons.

Few studies utilizing direct biochemical assays have been reported for the early nervous system. Cytospectrometric work has indicated that quantities of RNA increase steadily with development for the first five days in the cells of the neural tube and then fall at different rates in dorsal (sensory) and ventral (motor) regions (Hughes, 1955). Cells taken from chick embryos at 7 and 13 days have been studied for changes in energy metabolism as reflected by oxygen uptake, lactic acid production and content of ATP (Dittmann *et al.*, 1973). The rate of oxygen

uptake per cell doubled over the time studied and lactic acid production of the cell decreased. ATP per cell remained unchanged, as assayed by the luciferase method. The bulk of other studies on the developing nervous system which attempt to identify specific changes in cell proteins--enzymic and structural--utilize histochemical and immunologic techniques and are usually carried out on much older tissue. For example, acetylcholine esterase (AChE) and choline acetyltransferase (CAT) activities increase in parallel with morphologic differentiation of neuronal processes (Kim et al., 1974). Histochemical appearance of the enzymes was evident slightly later than the biochemical enzyme assays indicated. AChE appeared earlier than CAT, whose appearance corresponded to the development of synaptic components as determined with the electron microscope.

Little information is available in the literature which identifies the biochemical substrate of silver-staining (Sechrist, 1969) which has been suggested to represent the earliest evidence of neuronal differentiation, rather than the increase in endoplasmic reticulum suggested by Fujita and Fujita (1963), or the accumulation of neurofibrillary material (Lyser, 1964) as the axonal process begins to develop. Sechrist, in fact, claims that such evidence of neuron-specific differentiation is present in premitotic cells in the neural tube.

Gene Regulation in Nervous Tissue

We have suggested above that nervous tissue offers a unique opportunity to study the regulation and diversity of developmental processes within a single tissue with limited cellular proliferation and numerous specific gene products. The major types of studies which have been reported to date involve the differences in various regions of the brain

of the neurotransmitter enzyme systems with age or with experience or hormonal treatment. No direct investigations of the regulation of gene expression have been conducted, although studies have been made of the qualitative and quantitative composition of cytoplasmic proteins and of brain nuclei and brain chromatin. Transcriptional activity of brain chromatin has also been investigated. However, the heterogeneity of the material used in such studies prevents an unambiguous interpretation of the data.

Studies of brain chromatin rely heavily on methods of isolation of cell nuclei. Most published studies have started with homogenates of whole brain or of relatively large regions of brain. The complex structure of nervous tissue which consists of interdigitating cells of several size classes and degrees of branching, of cells joined by several types of specialized junctions, and satellite-type cells which do possess the capacity for movement and proliferation in addition to the more stable neuronal populations, introduces further complications and difficulties in both the design of experiments and the interpretation of data. In addition, attempts have been made to fractionate cell types or nuclear types prior to further characterization. A major drawback in all such studies is the lack of a reliable means of accurately identifying the composition of each fraction.

Studies of nervous tissue compare the attributes of various cell types, based on morphologic criteria such as large nuclei ("neuronal") and small nuclei ("glial"). cursory inspection of histological preparations of nervous tissue reveals the vast heterogeneity of cell (and nuclear) sizes. Hand dissection of neurons (Hyden and McEwen, 1966) has limited usefulness because of technical complexity, limited amounts

of material and sampling problems (only the largest neurons can be obtained). In non-mammalian vertebrates, there are populations of extremely large neurons which can be studied reliably in this fashion (Edstrom et al., 1969; Edstrom & Sjostrand, 1969).

Several bulk separation techniques have been applied to nervous tissue in attempts to separate cell types and isolated nuclear populations (Freysz et al., 1968; Satake et al., 1968; Rose, 1968; Rose and Sinha, 1969; Johnson and Sellinger, 1971; Sellinger et al., 1971; McEwen et al., 1972; McEwen and Zigmond, 1972; and Hadjiolov et al., 1965). However, Dounce's observation that "no single method at present is available for the isolation of all nuclei ..., in spite of implications by some investigators to the contrary" (1963, p. 127), is certainly still true and definitely applicable to nervous tissue.

Nuclear Protein Synthesis

Although protein synthesis is thought to occur exclusively in the cytoplasm, nuclear protein synthesis has been reported for several cell types, including neurons. Allfrey et al. (1964) reported that nuclei of thymocytes are capable of synthesizing protein by ribosome-dependent mechanisms which require amino acid activating enzymes, endogenous ATP, and soluble and stable RNA. This system was reported to differ from cytoplasmic protein synthesis in that it was resistant to RNase and required sodium, rather than potassium.

Burdman and Journey (1969) identified a similar system operating in nuclei isolated from rat brain. Incorporation of leucine was linear for 60 minutes, after which time the preparations showed "loss of stability." Both "neuronal" and "glial" fractions, separated on sucrose density gradients (Løvtrup-Rein and McEwen, 1966), incorporated leucine to different extents. In these nuclei, incorporation of amino acid decreased

with increased sodium and was inhibited by both puromycin and cycloheximide.

Two further studies of this phenomenon (Burdman et al., 1970; Burdman, 1972b) surveyed the incorporation of amino acid in vivo and in vitro into various fractions of proteins of the nuclei. These protein fractions were extracted with various concentrations of NaCl from intact nuclei and included "saline soluble," "residual acidic," "chromatin acidic," and histone fractions. The histones were found to be almost inert with respect to in vitro incorporation of leucine. "Residual proteins" were the most rapidly labelled, and "chromatin acidic proteins" had the highest activity. The ability of nuclei to incorporate such radioactivity decreased with age. Nuclei from adult animals had only 10% of the activity of nuclei from three-day-old rats. In contrast, when labelled leucine was administered to intact animals and brains were removed at various times up to four hours later, the time course of incorporation was similar in all age groups. Rates of incorporation could not be compared across ages since the amounts of label which were administered varied. This system was inhibited by hypertonic sucrose and required sodium only for some amino acids (not for valine, leucine, alanine or threonine). Actinomycin D had only a slight inhibitory effect and chloramphenicol had none.

Information from the age studies suggests that the purity of the nuclear fractions may have been different. Three-day-old rats have very little myelin, yet adult animals have large amounts, and this alone might be expected to alter the extraction efficiency. No mention is ever made of this problem in developmental studies.

Goldstein (1970) reviewed the evidence for nuclear protein synthesis

for many cell types. The major problems involved in most of the studies, protein migration from cytoplasm to nucleus and problems in nuclear isolation techniques, render conclusions about the possibility of nuclear protein synthesis impossible. Only two cell types in addition to neurons yield plausible data: isolated nuclei of thymocytes and pigeon erythrocytes. Both of these cell types have been considered atypical. The thymocyte has an unusually large nucleus with a small volume of cytoplasm and can produce ATP from glycolysis, the TCA cycle, and oxidative phosphorylation in the nucleus. It is involved in the immunologic recognition and defense system of the body and possesses a remarkable ability for specific differentiation. Perhaps the neuron, too, represents an "atypical" cell.

However, a study by Dravid and Wong (1972) may offer an explanation of neuronal "nuclear" protein synthesis. These authors noted that when sterile techniques were used, the amount of incorporation of amino acid decreased significantly. The incorporation measured in previous studies, therefore, was probably due to bacterial contamination. Caution must therefore be exerted in interpreting results of in vitro incubation procedures which do not use aseptic techniques.

In spite of this study emphasizing the importance of sterile techniques, no reference is made to this work in the later studies of Burdman and coworkers (Burdman, 1972 a, b; Burdman et al., 1973; Szijan and Burdman, 1973 a, b; 1974). A recent study of nuclear protein synthesis following in vitro incorporation of isolated nuclei from brain (Fleischer-Lambropoulos and Reinsch, 1975) made no mention of the use of sterile techniques or of possible contamination of nuclear fractions either with bacteria or with cytoplasmic components.

In summary, no definitive evidence yet exists which suggests that neurons differ from other cell types in their dependence on the cytoplasm as the site of protein synthesis, including proteins which may be involved in the regulation of nuclear events.

Brain Nuclei and Brain Chromatin

Studies which were directly designed to study characteristics of the genome and its regulation in brain tissue can be grouped into several categories: 1) composition and properties of isolated chromatin and nuclei from brain (see Tables 1-3); 2) differences in the spectrum of nuclear proteins in brain versus other tissues; 3) age-related changes in brain nuclear proteins; 4) region-related differences in nuclear proteins of brain; 5) experience- and sex-related differences in such proteins; 6) alterations in RNA synthesis (template activity or hybridization properties) with age or regional differences; and 7) hormonally induced events in the nucleus.

Interpretation of the results of almost all the studies is limited by problems resulting from the use of whole brain homogenates (or homogenates of large regions of brain) as a source of nuclei or chromatin. Numerous cell types contribute to such fractions. Reflections of such heterogeneity can be seen in the range of values which have been reported for various measurements made on "clean" nuclear preparations (Table 1) or on "chromatin" (Table 2). These problems are further compounded by the variations in methodology used to prepare such fractions and to separate out the various groups of proteins. No well-designed studies have yet systematically compared the major methods in use to determine ways to compare such results. Because of these problems, it is most useful to summarize such studies of brain nuclear proteins in a very

TABLE 1

Nuclei from brain: mass ratios of protein and RNA to DNA

| Source | Protein/DNA | Histone/DNA | Nonhistone/DNA | RNA/DNA | Reference |
|-------------------|-------------|-------------|----------------|-----------|--------------------------------|
| Adult cat cortex | 0.26-0.37 | | | 0.50-0.52 | Hadjiolov <u>et al.</u> , 1965 |
| Rat cerebellum | 2.2 | 0.70 | 1.63, 0.23 | 0.011 | McEwen <u>et al.</u> , 1972 |
| other | 4.8 | | | | |
| hippocampus | | 0.86 | 2.54, 1.56 | 0.18 | |
| med. + post. ctx. | | 1.05 | 2.64, 1.99 | 0.19 | |
| amygdala + ctx. | | 0.99 | 2.49, 2.29 | 0.20 | |
| Rat brain | | | | | Austoker <u>et al.</u> , 1972 |
| *Fraction I | 5.20 | | | 0.60 | |
| II | 5.14 | | | 0.66 | |
| III | 4.32 | | | 0.49 | |
| IV | 2.47 | | | 0.36 | |
| V | 2.40 | | | 0.32 | |
| Rat brain | 2.2-7.5 | | | 0.08-0.45 | McEwen and Zigmund, 1972 |
| Rat brain | 2.5-3.5 | | | | Dravid and Wong, 1972 |
| Rat brain | 3.24 | | | | Duerre and Gaitonde, 1971 |
| Rat brain | 5.10 | | | | Piha and Jokela, 1972 |
| Rat cortex | | | 3.12 | | Fujitani and Holoubek, 1974 |
| cerebellum | | | 2.20 | | |
| rest | | | 2.73 | | |
| Rat hippocampus | 5.38 | | | 0.292 | Fleischer-Lambropoulos and |
| cortex | 5.01 | | | 0.263 | Reinsch, 1975 |
| medulla | 4.21 | | | 0.204 | |
| cerebellum | 2.97 | | | 0.105 | |
| rest | 4.47 | | | 0.249 | |

*These fractions have different compositions with respect to size groupings of nuclei.

TABLE 2
Brain chromatin: mass ratios of protein and RNA to DNA

| Source | Protein/DNA | Histone/DNA | Nonhistone/DNA | RNA/DNA | Reference |
|----------------------------------|---------------------------|--------------------------------|----------------------|--------------------------------|---|
| Chicken embryo-4 day 8 day | 2.8-3.2 2.9-3.0 | 0.90-0.91 0.88-0.99 | | 0.28-0.30 0.19-0.24 | Dingman and Sporn, 1964 |
| Adult hen | 1.6-2.1 2.7-3.1 1.8 | 0.78-0.93 0.76-0.80 0.72 | | 0.08-0.12 0.17-0.19 0.08 | |
| Mouse | 1.8-4.5 | | | | Kurtz and Sinex, 1967 |
| Chicken embryo-11 day | 3.45 | | (1.04-1.48) | | Graziano and Huang, 1971 |
| Pig cerebellum pituitary | | 1.60 1.56 | 0.50 0.45 | 0.13 0.108 | Shaw and Huang, 1970 |
| Rat large nuclei small | 3.9 1.5 | | | | Olpe <u>et al.</u> , 1972 |
| Rat | | 1.42 | 0.84 | | Duerre and Gaftonde, 1971 |
| Newborn rat | | 0.85-1.14 | 0.81-1.46 2.31 | 0.06-0.10 0.07-0.08 | Bondy and Roberts, 1969 |
| Rat cortex cerebellum rest | | | 0.52 0.18 0.38 | | Fujitani and Holoubek, 1974 |
| Ox cortex | 1.92 | | | 0.09 | Singh and Sung, 1972 |
| Rat | 1.79 | | | | Piha and Jokela, 1972 |
| Rat | | 1.1 | | | Duerre and Lee, 1974 |
| Female rat-neuronal glial | 1.86 1.48 | 0.91 0.86 | 0.95 0.62 | | Fleischer-Iambropoulos <u>et al.</u> , 1974 |

TABLE 3

Thermal stability of brain chromatin

| Source | T _m | T _m Standard DNA | Reference |
|----------------|----------------|-----------------------------|-------------------------|
| Chicken | 76-79 | 41-42 | Dingman and Sporn, 1964 |
| Rat cerebellum | 76.5 | 43 | Shaw and Huang, 1970 |
| pituitary | 77.1 | | |
| Rat | 77 | 59 | Bondy and Roberts, 1969 |

general fashion. Studies involving interactions of hormones with nervous tissue most nearly approach the ideal situation.

In general, histones of brain are identical to those of various other tissues. All the histone classes are present and vary very little across different regions of the brain or with age (Dingman and Sporn, 1964; Bekhor et al., 1964; and Duerre and Gaitonde, 1971). They appear to be the most stable components of brain nuclei--and, in fact, are almost inert with respect to new synthesis or turnover in both short and long term studies (Piha et al., 1966; Burdman et al., 1970, 1973; Bondy, 1971; Burdman, 1972). This is consistent with the tight coupling of histone synthesis with DNA synthesis which is thought to occur in all tissues of multicellular organisms. Relatively little DNA synthesis and/or cell division occurs in mature nervous tissue. Enzymes which modify histones, such as acetylases (Caspary and Sewell, 1968; Bondy et al., 1970) and methylases (Miyake and Kakimoto, 1973; Duerre and Lee, 1974), have been described for brain.

The nonhistone proteins of brain, in contrast, appear to differ in their distribution on polyacrylamide gels from those of various other tissues of the body (Dingman and Sporn, 1964; Bekhor et al., 1964; MacGillivray et al., 1972; Uyemura, 1974; and Elgin, 1972) to varying degrees depending on the methods used to isolate and fractionate them. Although Burdman and coworkers claim that these proteins, as well as the histones, can be synthesized in isolated brain cell nuclei, it is more likely that they are synthesized in the cytoplasm and then migrate into the nucleus (see the previous section on nuclear protein synthesis). The nonhistone proteins as a group appear to turn over much more rapidly than do the histones (Burdman et al., 1973) and are very heterogenous

(Graziano and Huang, 1971; Piha and Jokela, 1972; Jokela and Piha, 1972; Davis et al., 1972; Olpe et al., 1972, 1973; Kohl et al., 1973). When comparisons are made between nonhistone proteins from young animals (usually within a few days of birth as the earliest age examined) and from adults, some differences are seen both in the spectrum of proteins present and in their rates of labelling following in vivo injections of isotopically labelled amino acids (Kurtz and Sinex, 1967; Dravid and Burdman, 1968; Shaw and Huang, 1970; Szijan and Burdman, 1973b; and Biessmann and Rajewsky, 1975). These differences have been related to the various degrees of development or activity of the brain regions involved; however such results should be looked at much more closely. Many components of nervous tissue, especially non-neuronal ones (glia, blood vessels, choroid, and meninges), vary with age and may affect isolation and fractionation procedures.

It has also been suggested that alterations in the composition of nonhistone proteins (or in post-translational modifications of them) may reflect differences in neurons versus glial cells (Fleischer-Lambropoulos et al., 1974) in amount or type of experience (Glassman et al., 1972) or in sex of the animals (Yu, 1975). Although Yu found no differences in several regions of brain in the histones or nonhistone proteins among male, female or neonatally androgenized female rats, it was suggested that such differences do, in fact, exist but the capacity of the gel system or of the extraction procedure to detect such differences was insufficient.

Nonhistone nuclear proteins of brain include various types of enzymes, such as RNA and DNA polymerases (Singh and Sung, 1972; Szijan and Burdman, 1974) as in other tissues (Elgin et al., 1971).

DNA-dependent RNA synthesis in brain, using either endogenous polymerase or E. coli polymerase, has been reported to be both higher and lower than in other tissues (Dingman and Sporn, 1964; Bondy and Roberts, 1969; Smith et al., 1969; Austoker et al., 1972; Singh and Sung, 1972; Burdman et al., 1973). These values, if lower than other tissues, are interpreted to reflect the non-proliferating nature of nervous tissue; and, if higher than other tissue, to reflect the greater number of genes or more active genome being expressed in nervous tissue. The differences are interpreted therefore to reflect functional differences in the chromatin.

Most preparations of chromatin from cerebellum seem to differ significantly in content and transcriptional properties from other regions of the brain (see Tables 1 and 2). This has been interpreted to reflect the less active gene expression in this region of the brain, perhaps because the preparations are more homogeneous. Isolated nuclear fractions from cerebellum seem to be made up mostly of small-sized nuclei, perhaps from granule cells which are numerous and densely packed in this region of brain.

Hormones are thought to act very specifically in certain areas of the brain to influence developmental and metabolic events which are reflected in behavioral and sexual differences in animals. Several laboratories are investigating the steroid binding properties of the hippocampus and hypothalamus (McEwen et al., 1972; Chytil and Toft, 1972; and Grossner et al., 1973). It is thought that such hormones are taken up specifically by certain cells, bound by cytoplasmic receptor molecule(s), and transferred to the nucleus where they interact with DNA-associated proteins (nonhistones) to selectively alter gene expression.

Alterations in RNA and protein synthesis have also been reported following hormone administration (Garfield and Moscona, 1973) to chick neural retina. Studies in non-neural tissues include those of Mauer and Chalkley, 1967; O'Malley et al., 1968; Cox and Carey, 1971; and Comstock et al., 1972). Other studies in non-neural tissue, such as the chick oviduct, suggest that the hormones interact selectively with nonhistones to initiate changes (Tucker et al., 1971; Spelsberg et al., 1972a; Stein et al., 1974; and O'Malley and Means, 1974).

Studies of pituitary cells in culture (Watanabe et al., 1973 a, b) have demonstrated similar effects of steroids on the ACTH-secreting capacity of these cells. Specific hormone receptor molecules are being sought. These studies, using a cultured tumor cell line, are particularly interesting since they circumvent many of the problems we have mentioned which occur in the use of intact brain tissue.

Neuroblastoma C1300 Cell Lines

In addition to the neural retina and pituitary cells in vitro mentioned in the previous section, several continuous lines of nervous system-like cells have been developed, mainly from mouse tumors. These cell lines are designated glioma or glioblastoma and neuroblastoma to indicate their similarity to and probable origin from glial and neuronal cells, respectively. These cell lines have been extensively studied and compared to normal nervous tissue (see McMorris et al., 1973; Sato, 1973; and McMorris and Ruddle, 1974), and have "helped bring the technology of molecular and cellular biology to the neurosciences" (Haffke and Seeds, 1975, p. 1655).

The origin of the original mouse tumor from which the cultured neuroblastoma cell lines have been derived, and with which we will be

concerned, is somewhat obscure. The earliest reference to the C1300 mouse tumor in the literature appears to be that of Gorer (1947) who used the tumor in a study of the antibody response in mice to inoculated tumor cells. The C1300 tumor was described as a "round cell tumor, possibly a neuroblastoma." Additional early references to the C1300 tumor are useful to review since they describe the growth characteristics of the tumor in vivo and the apparent lack of knowledge of the origin of the tumor--in spite of which it has been readily accepted as a neuronal model system and often reported to be of neuronal origin by recent investigators.

Snell et al., (1948) described the C1300 as a "neurogenic sarcoma, A strain origin." Eichwald and coworkers (Eichwald et al., 1950; Eichwald and Chang, 1951) transplanted the tumor into the anterior chamber of the eye of C57Brown and ABC mice and observed different degrees of metastases in the different host strains. Moore (1951) reported that the C1300 tumor arose in the abdominal cavity of an A strain mouse and when transferred into homologous strains "took" 100% of the time. Tumors became palpable in the new host animals in seven days and grew until the host died at four to five weeks. The tumor contained closely packed round cells. This tumor was also noted to be the most susceptible of the five tumor types tested to the Russian Far East encephalitic virus.

Klein (1951) reported on an extensive study of mouse tumors and stated that tumor C1300 was found by Dr. Clondman at the Jackson Laboratory in 1940 as "a huge irregular red and white tumor filling the abdominal cavity of an A strain mouse." In these studies the tumor was transplanted subcutaneously and appeared as a diffusely growing anaplastic tumor with mainly polyhedral and round cells. After interperitoneal

inoculations, huge solid tumors developed and the hosts survived for a median time of twenty days. The tumor was unique among those tested in that it followed two clinical courses: either to produce a solid tumor or bloody exudate.

Dunham and Stewart (1953) in an extensive summary of transplantable and transmissible animal tumors listed the C1300 as a tumor of nervous tissue, with the comment, "Classification in doubt: 'possibly a neuroblastoma.'" Hauschka and coworkers (1956) reported that the modal chromosome number, based on unpublished data of Dr. A. Levan, was 66 to 70 (the diploid value for the mouse is 40).

From this short review it is apparent that the C1300 tumor appeared spontaneously in the mouse A strain. Schubert and coworkers (1973) discuss possible alternatives which would account for the heterogeneity which is observed both in the tumor behavior and in studies, to be discussed below, of cloned cell lines in vitro. The most simple explanation that they feel is consistent with observations on other neoplasms and cell lines is that the tumor was originally derived from a single neoplastic event and subsequently underwent alterations in the genetic material during the thousands of cell generations in vivo and in vitro. The variations in phenotype seen in the various clones from the tumor probably all represent activities present in the original neoplastic cell which have been selectively lost in some derivative lines.

Three labs simultaneously reported the in vitro culture of the C1300 tumor and proposed its usefulness as a model of neuronal cells, in much the same way as Murray and Stout (1947) had two decades before for human neuroblastoma (Augusti-Tocco and Sato, 1969; Klebe and Ruddie, 1969; and Schubert et al., 1969). Mouse tumor cells in suspension cultures

were described as round cells (like the in vivo tumor), 40 μ in diameter with one to three nucleoli, diffuse endoplasmic reticulum, and associated virus particles. The chromosome complement was described as being approximately tetraploid. In contrast, cells cultured in conditions which allowed attachment to a substrate developed into monolayers with large (40 to 140 μ diameter cell bodies) flattened cells with one to eight nucleoli and processes extending out from the cell body for two to three millimeters. In addition, cells in the monolayer cultures stained with Bodian silver techniques and seemed to have fewer virus particles. Clones derived from the original tumor cells in culture were reported to have measurable activities of tyrosine hydroxylase (TyH), choline acetyltransferase (CAT), acetylcholine esterase (AChE) and microtubular protein.

An amazing number of studies has been reported dealing with the morphological characteristics (Augusti-Tocco et al., 1973; Schubert et al., 1969; DeLellis et al., 1970; Seeds et al., 1970; Nelson et al., 1971 a, b; Prasad and Hsie, 1971; Prasad, 1971 a, b, 1972 d; Prasad and Sheppard, 1972; Prasad et al., Byfield and Karlsson, 1973; Chang and Goldman, 1973; Ross et al., 1973; Daniels and Hembrecht, 1973, 1974; Hinckley and Telsner, 1974; Braskefield et al., 1975; Ross et al., 1975), electrophysiological properties (Harris and Dennis, 1970; Nelson et al., 1969, 1971 a, b; Peacock et al., 1972; Nelson, 1973; Schubert et al., 1973; Spector et al., 1973), surface components (Brown, 1972; Augusti-Tocco et al., 1973; Schachner, 1973; Truding and Morell, 1973; Kimelberg, 1974; Stefanovic et al., 1974; Akeson and Herschman, 1974 a, b; Matthews et al., 1976), nucleic acid metabolism (Schubert and Jacob, 1970; Seeds et al., 1970; Rosenberg et al., 1971; Prasad et al.,

1972, 1973; Rosenberg, 1973; Augusti-Tocco et al., 1973; Bondy et al., 1974), and general protein synthesis (Schubert and Jacob, 1970; Schubert et al., 1973) of various clonal lines of the C1300 tumor under several conditions of culture (see Schrier et al. 1974, for list of clones and their properties).

Early in the tissue culture work, as mentioned above, it was noted that the morphology of the cells, as well as biochemical characteristics, shifted toward a more neuron-like spectrum when the cells were shifted from suspension to monolayer culture (Schubert et al., 1969; Olmsted and Rosenbaum, 1969; Olmsted et al., 1970; Schachner, 1973; Augusti-Tocco et al., 1973; Casola et al., 1974) or were treated with various agents, including exposure to X-ray (Prasad 1971 a, b, 1972 b, d; Prasad and Mandal, 1972, 1973; Prasad et al., 1972), bromodeoxyuridine (Schubert and Jacob, 1970; Land and Goldstein, 1971; Brown, 1972), prostaglandin E₁ (Gilman and Nirenberg, 1971; Prasad, 1972 a, b, d, e; Prasad and Mandal, 1972; Sheppard and Prasad, 1973; Sahu and Prasad, 1975), dibutyryl cyclic AMP (Furmanski et al., 1971; Prasad, 1972 b, c, d; Prasad et al., 1972 b, 1973; Waymire et al., 1972; Prasad and Gilmer, 1973; Prasad and Mandal, 1972; Sheppard, 1972; Truding and Morell, 1973; Chalazonitis and Greene, 1974; Yavin et al., 1975), phosphodiesterase inhibitors (Gilman and Nirenberg, 1971; Prasad, 1972 b; Sheppard and Prasad, 1973; Bondy et al., 1974), removal of serum from the medium (Seeds et al., 1970; Kates et al., 1971; Ciesielski-Treska et al., 1972; Furmanski and Lubin, 1972; Hermetet et al., 1972 a, b; Nissen et al., 1972, 1973; Sheppard and Prasad, 1973; Stefanovic et al., 1974; Akeson and Herschman, 1974 a, b; Yavin et al., 1975), or exposure to hypertonic media (Ross et al., 1973; Rosenbaum, 1973).

Numerous other compounds have also been used (Kates et al., 1971; Miller and Levine, 1972; Peacock et al., 1972; Prasad, 1972 d; Furmanski and Lubin, 1972; Prasad and Sheppard, 1972; Byfield and Karlsson, 1973; Schneider, 1974; Hinckley and Telser, 1974; Kimhi et al., 1976). Such alterations in the biochemical and morphological characteristics of the neuroblastoma cells have been likened to neuronal differentiation; however, Schubert and coworkers (1973) discuss the problem of describing the changes in the neuroblastoma cells, involving neurite extension, as differentiation. The alterations are often reversible, and there are great difficulties in deciding whether the concept of "cellular differentiation" is appropriate. As discussed by Weiss (1939) and Grobstein (1959), "differentiation" refers to an irreversible, terminal process. Because of the semantic and conceptual problems with the use of the word "differentiation" and comparison of the phenomenon in vivo and in vitro, we will use "differentiation" to refer to the alterations in neuroblastoma cells under different culture conditons and define it as Schubert et al. (1973) do: "a process directed toward a given end but not necessarily reaching that final stage of ultimate functional complexity and specialization," which is perhaps closer to the concept of modulation discussed by Weiss (1939) to indicate properties which appear in response to an external alteration in the cellular environment and which may be reversible when that alteration is reversed. Most of the studies discussed here use the extension of one or more processes at least as long as the diameter of the cell body as a criterion of "differentiation."

Many of the studies mentioned above, and others, have investigated

the activity of enzymes which are involved with neurotransmitter synthesis or degradation in normal nervous tissue. As a general finding, levels of activity of these enzymes increased when the cells were grown under conditions which induced morphological "differentiation." Clones with various combinations of catecholaminergic (TyH: Augusti-Tocco and Sato, 1969; Amano et al., 1972; Schubert et al., 1969; Waymire et al., 1972; Breakefield et al., 1975; aromatic L-amino acid decarboxylase: Schubert et al., 1969; monoamine oxidase and catechol-O-methyl transferase: Blume et al., 1970; Prasad and Mandal, 1972) and cholinergic (AChE: Blume et al., 1970; Kates et al., 1971; Hermetet et al., 1972 b, c; Rosenberg, 1973; Schubert et al., 1973; Lanks et al., 1974; CAT: Rosenberg et al., 1971; Prasad and Mandal, 1973; Steinbach et al., 1974) enzymes were found, as well as either enzyme group alone. Serotonergic systems seem to be less active in these cells, but are present (Knapp and Mandell, 1974).

In addition, neurotransmitter-sensitive adenylylase systems are also present in various clones (Prasad and Gilmer, 1973; Sahu and Prasad, 1975) similar to the ones which have been described in normal nervous tissue (Von Hungen and Roberts, 1974).

Although the "differentiated" neuroblastoma cells have been reported to have electrophysiological properties similar to those of normal neurons (see references above), including tetrodotoxin-sensitive action potential generating systems, only one laboratory has reported ultrastructural evidence of synapses between adjacent cells (Nelson et al., 1971 a, b). In fact, in more recent work (Ross et al., 1973, Rosenbaum,

1973) it has been pointed out that only when the cells are grown in hypertonic media are junctions between cells and vesicles indistinguishable from synaptic vesicles visible. Breakefield and coworkers (1975) have recently investigated an "adrenergic clone" (with TyH and dopamine B-hydroxylase) which appears to have catecholamine storage granules identical to those of normal neurons.

Several studies of glucose metabolism and oxygen uptake have been reported which suggest that, like maturing neurons, "differentiating" neuroblastoma cell cultures take up oxygen and rely more on aerobic than anaerobic metabolic pathways than do the "non-differentiating" cultures (Nissen et al., 1972, 1973; Ciesielski-Treska et al., 1972; Tholey et al., 1974; Rosenberg, 1973; Sakamoto, 1971; Sakamoto and Prasad, 1972).

Neuroblastoma cells are able to synthesize 14-3-2 protein (Herschman et al., 1973) which is thought to be a neuron-specific protein (Moore, 1973). The synthesis of phospholipids (Eichberg et al., 1975), fatty acids (Yavin et al., 1975), glycosphingolipids and glycosaminoglycans (Stoolmiller et al., 1973) and phosphoproteins (Casola et al., 1974) have also been studied in neuroblastoma clones.

The characteristics of heterokaryons (cells with a nucleus resulting from nuclear fusion, see Harris, 1970, and Ephrussi, 1972) from fusion of neuroblastoma cells with mouse L cells (fibroblast type) or glioma cell lines have been studied by several laboratories (Nelson, 1973; Minna et al., 1971; Minna, 1973; Daniels and Hambrecht, 1973, 1974; McMorris and Ruddle, 1974; Amano et al., 1974; Sharma et al., 1975) or between mouse neuroblastoma cells and sympathetic ganglion cells (Chalazonis et al., 1975; Green et al., 1975). In general, characteristics (enzyme content, electrophysiological properties) have been demonstrated

in the heretokaryons which are absent in the non-neuroblastoma parent cell line. Attempts have been made to interpret such events in terms of the regulation of expression of the genes of the two parent cell lines or to correlate certain characteristics with retention of specific chromosomes (chromosomes of the slower-dividing parent cell line tend to be lost with successive heterokaryon generations). However, the karyotypes of the parent lines themselves are extremely variable, especially after many generations in culture (Ciesielski-Treska et al., 1975). Shannon and Macy (1972) characterize the chromosome number of the Neuro 2a clone (available from the American Type Culture Collection) as 59 to 193 based on an analysis of 53 cells in one culture (A/J mice normal karyotype = 40).

Another series of studies deals with the interaction of neuroblastoma cells with other tissue culture cell lines. For example, Schubert and coworkers (1973) describe studies in which neuroblastoma cells are grown with myoblasts and Monard and coworkers (1973 a, b) report differences in the ability of other cells to induce "differentiation" of the neuroblastoma cells either when grown with them or when neuroblastoma cells are exposed to conditioned media in which the other cell types have been grown. Great caution should be exercised in interpreting the results of these studies. Methodological problems, such as depletion of factors in the conditioned media which would then act in a manner similar to serum-free media to induce "differentiation," are difficult to avoid. In addition, the other cell lines used are usually ones which, if not originally neoplastic themselves, have been in culture for many generations and may be altered from their in vivo tissue of origin.

The neoplastic nature of model systems, including the neuroblastoma

one, cannot be overlooked. The ability of these cell lines to proliferate indefinitely, their neoplastic origin, and their aneuploidy argue for the abnormality of regulatory processes basic to the survival of these cells both in vivo and in vitro.

To describe all the combinations of enzyme activities, susceptibility of neurite extension and/or increases in enzyme activity to various inhibitors, the spectrum of generation times, or the time course of neurite development which have been reported in the literature requires more space than is available here. All of these characteristics vary to some extent from clone to clone. Perhaps two characteristics of the cells would be useful to be used as early neuron-specific markers. These are (1) cell surface properties, especially nervous-tissue specific antigens (Akeson and Herschman, 1974 a, b) and (2) microtubule protein which is thought to be involved in the extension and maintenance of the processes (Olmsted and Rosenbaum, 1969; Olmsted et al., 1970). Process formation, which is the usual criterion used to identify "differentiated" cells, seems to be correlated consistently with the surface antigens to a much greater degree than with any other characteristic assayed. Therefore, it seems reasonable to use the development of the surface antigen or of microtubule protein as an indication of specific gene expression. A major problem, however, involves the difficulty in obtaining sufficient material to analyze the synthesis of a specific protein and the potential instability of the cell clones when cultured through the number of generations necessary to obtain sufficient quantities of material to carry out such an analysis. We will probably have to be content with less complete studies until our methods are refined sufficiently to allow subcellular fractionation on a microscale.

Although the neuroblastoma cell lines have been compared to normal adult brain tissue (Amano et al., 1972) and to fractions of brain tissue thought to be enriched for neuronal-type cells (Kimelberg, 1974), no effort has been made to compare them with cells from the early stages of nervous system development. The studies of early neural tube in vivo and in vitro provide an ideal "system to determine the degree of similarity between the tumor-derived cell lines and normal brain tissue (Kim and Wenger, 1972 a, b; Kim et al., 1974).

To date no systematic study of the regulation of the nuclear processes involved in the induction and maintenance of "differentiation" in the mouse neuroblastoma cells has been conducted. We have therefore undertaken an investigation and comparison of nuclei isolated from neuroblastoma cells grown under conditions in which the majority of cells are either round and dividing or neuron-like with limited division and branching processes.

CHAPTER II

METHODS

Cell Culture

Neuro 2a clone of mouse neuroblastoma C1300 cells were obtained from the American Type Tissue Collection and were maintained in mono-layer culture in minimal essential medium (Eagle) with Earle's salts and non-essential amino acids (MEM), 50 U/ml penicillin-G (potassium salt), 10 µg/ml streptomycin sulfate and 10% fetal calf serum (FCS) (vol/vol). Cells were grown in plastic tissue culture flasks or 150 mm Petri dishes (Falcon Plastics) or in one-liter glass Blake flasks (Bellco Glass). Medium was changed every three to four days and cells were subcultured with 0.05% trypsin (wt/vol) in calcium- and magnesium-free Earle's balanced salt solution. Tissue culture medium and serum were purchased from Grand Island Biological Company.

Stocks of cells were frozen in liquid nitrogen for storage in MEM supplemented with 20% FCS (vol/vol) and 5% glycerol (vol/vol).

Cell Growth and Differentiation

To determine cell growth characteristics, 25 cm² plastic flasks were inoculated with approximately 10⁵ cells in MEM with 10% FCS. After an adequate number of cells had attached, usually within two hours, five ml of new medium containing 0, 10, or 20% FCS or 10% FCS with 0.5 or 1.0 mM N⁶, O^{2'}-dibutyryl adenosine 3':5'-cyclic monophosphoric acid (db cAMP; grade II, sodium salt, Sigma Chemicals) were added. Cells were counted every two to six hours in the same three fields in each flask.

Fields were identified by means of a black plexiglass template which fitted over the flask. Cell number and the percentage of cells with at least one process as long as the cell diameter ("differentiated" cells) were determined. Counting of cells was carried out in a 37° C warm room to minimize the effect of temperature fluctuation on cell growth.

After 24 hours, medium in flasks from each growth condition was replaced with fresh MEM plus 10% or 20% FCS and incubated for an additional 24-hour period.

The viability of attached cells and cells in suspension was assessed by the trypan blue dye exclusion method (Paul, 1970). Cells were incubated for five minutes in 1% trypan blue (wt/vol) in balanced salt solution and the percentage of cells excluding the dye was determined by standard hemacytometer counting methods.

For morphological studies, cells were fixed in situ in the plastic flasks or were grown on glass slides in Petri dishes. Cultures were washed free of medium with balanced salt solution and were then fixed with one of several fixatives: acetic acid-ethanol (1:3), 2% glutaraldehyde (vol/vol) in 0.1 M sodium phosphate buffer (pH 7.6), 10% phosphate-buffered formalin (vol/vol), formalin-acetic acid-ethanol (5:5:72), or 2% ammonium bromide (wt/vol) -6% formalin (vol/vol). Cells were then stained with one of several cell stains: hematoxylin, toluidine blue, cresyl violet or Giemsa (Armed Forces Institute of Pathology Manual). In addition, three modifications of the Bodian silver protargol method specific for neurons was used (Kim, 1971; J.J. Bernstein, Department of Neuroscience, University of Florida, personal communication--see Table 4; Sevier and Munger, 1965).

Human diploid fibroblasts (ATCC 1121, a gift of Dr. Joyce Rensen,

TABLE 4

Modification of Bodian silver protargol staining technique (J.J. Bernstein, Department of Neuroscience, University of Florida) for tissue and cells on slides.

1. Hydrate slides and wash twice in distilled water.
2. Place in 1% protargol solution^a for 24 hours at 37° C.
3. Place in reducing solution^{b,c} with agitation for 1 minute.
4. Rinse in running distilled water for several minutes.
5. Place in 1% gold chloride (wt/vol) for 2 minutes.
6. Wash in distilled water for 1 minute.
7. Place in 2% oxalic acid (wt/vol) for 5 minutes.
8. Wash in two changes of distilled water for 1 minute each.
9. Place in 5% sodium thiosulfate (wt/vol) for 4 minutes.
10. Rinse in running distilled water for at least 5 minutes.
11. Counterstain with cresyl violet or directly dehydrate through 100% ethanol (vol/vol).
12. Place in xylene:ethanol (equal volumes) for 1 minute.
13. Place in two changes of xylene for 1 minute each.
14. Mount coverslip.

^aPlace slides in staining dish in distilled water. Sprinkle protargol on surface of the water (1 g/100 ml). Do not mix. Protect from light.

^bReducer is made fresh for each staining dish. Combine solutions A, B, and C immediately before use. Solution A: 4.8 g gelatin in 160 ml Pearson O'Neil buffered water; solution B: 0.08 g silver nitrate in 40 ml distilled water; solution C: 0.16 g hydroquinone in 16 ml Pearson O'Neil buffered water.

^cPearson O'Neil buffered water (pH 4.1): 14 ml of 0.01% acetic acid (vol/vol)--0.003% sodium acetate (wt/vol) solution plus distilled water to make one liter.

Department of Biochemistry, University of Florida) and frozen sections of rat brain were used for comparison with the neuroblastoma cells.

To compare the Neuro 2a clone with another cloned line of mouse neuroblastoma cells, clone 2 (D₁) was obtained from Dr. Jack Waymire (Department of Psychobiology, University of California, Irvine). These cells were grown on slides in Ham's F12 medium with 10% FCS.

Preparation of Nuclei and Chromatin

Nuclei and chromatin were prepared at 4° C following mechanical harvesting of cells with a rubber policeman from Blake flasks or Petri dishes according to procedures described by Stein and Borun (1972) and Stein and Thrall (1973). Cells were washed three times with Earle's balanced salt solution and lysed with 80 mM NaCl, 20 mM EDTA, 1% Triton X-100 (vol/vol) at pH 7.2. Nuclei were pelleted by centrifugation at 1000 x g for five minutes and washed three times with the lysing medium. Nuclei were then washed twice with 0.15 M NaCl in 0.01 M Tris at pH 8.0. Isolated nuclear preparations were examined with phase contrast microscopy.

Cell lysis and washing of nuclei were carried out both in the presence and absence of L-t-tosylamide-2-phenyl-ethyl-chloromethyl-ketone (TPCK, Sigma Chemicals), 50 µg/ml, to inhibit proteolytic degradation (Taber et al., 1973). Comparisons were made of the molecular weight profiles of nuclear and chromatin proteins (as described below) prepared with and without the inhibitor.

Nuclei were prepared for electron microscopic examination following centrifugation at 1000 x g by fixation in 2% osmium tetroxide (vol/vol) in 0.1 M sodium cacodylate, pH 7.4 for 30 minutes, washed, and rapidly dehydrated in ethanol. Nuclei were then infiltrated for several hours

and embedded in Spurr's low viscosity medium (Spurr, 1969). One micron sections were stained with 0.1% toluidine blue (wt/vol) containing 1% sodium borate (wt/vol). Thin sections were stained with 7.5% uranyl acetate (wt/vol) in 50% ethanol (vol/vol) (Watson, 1958) and Sato's lead stain (Sato, 1968) and examined with a Siemens 1A electron microscope. Tissue blocks were sectioned and examined by Dr. Kelly Selman (Division of Anatomy, Department of Pathology, University of Florida).

Chromatin was prepared from isolated nuclei by lysis in distilled water with gentle homogenization with a Dounce glass homogenizer and "A" pestle. The nuclear material was allowed to swell in an ice bath for 30 minutes and was then pelleted by centrifugation at $20,000 \times g$ for 15 minutes.

Possible contamination of chromatin by cytoplasmic proteins during the harvesting and isolation procedures was examined by mixing the cytoplasmic fraction prepared from cells labelled for 30 minutes with L-leucine-[4,5- 3H] (5 $\mu Ci/ml$ final concentration, New England Nuclear) prior to harvesting with nuclei isolated from unlabelled cells. Chromatin was then prepared as described above. The amount of tritium in the final chromatin preparation was compared to that added to the unlabelled nuclei.

In vitro Transcription of Chromatin

The ability of chromatin from neuroblastoma cells grown in medium containing 0 or 10% FCS or 10% FCS plus 1.0 mM db cAMP to support RNA synthesis in vitro was determined using *E. coli* RNA polymerase prepared according to the procedure of Burgess (1969). Chromatin was resuspended in 0.01 M Tris (pH 8.0) by several strokes with a Teflon pestle in a glass homogenizer and dialyzed against 1000 volumes of 0.01 M Tris (pH 8.0) for 12 hours at 4° C. Assay of RNA synthesis was carried out by

the method of Bonner et al., (1968) or by a modification of the method of Murphy et al. (1973).

Using the method of Bonner et al., the reaction mixture, in a final volume of 250 μ l, contained 20 μ l of *E. coli* RNA polymerase, 0.02 mole of sodium phosphate (pH 7.0), 1 μ Mole $MgCl_2$, 0.25 μ Mole $MnCl_2$, 3 μ Moles 2-mercaptoethanol, 0.1 μ Mole each of guanosine 5'-triphosphate (GTP), cytidine 5'-triphosphate (CTP), and uridine 5'-triphosphate (UTP). 0 to 180 μ l of chromatin (or equivalent amounts of calf thymus DNA), and 0.1 μ Ci of adenosine 5'-triphosphate-tetrasodium-[8- ^{14}C] (^{14}C -ATP, 44 mCi/mM, Schwarz Mann). After incubation at 37° C for ten minutes, the reaction was stopped with the addition of four to five ml of cold 10% trichloroacetic acid (wt/vol, TCA). The precipitates were collected after five minutes on presoaked Millipore filters (HA 0.45 μ) and washed three times with cold 10% TCA. The filters were air dried and dissolved in 1 ml of Cellusolve (ethylene glycol monoethyl ether) and counted in 15 ml of scintillation cocktail containing Cellusolve, toluene and Liquifluor (New England Nuclear) in a ratio of 1:3:0.16.

In a later experiment, the substrate cocktail was modified to omit the phosphate and contained 4 mMoles $MgCl_2$, 1.0 mMoles $MnCl_2$, 0.02 mMoles EDTA (disodium salt), 0.009% 2-mercaptoethanol (vol/vol), 0.4 mMoles each of GTP, UTP, and CTP and 0.1 μ Ci ^{14}C -ATP.

Using the modified method of Murphy et al. the reaction mixture, in a final volume of 750 μ l, contained 10 μ l of *E. coli* RNA polymerase, 50 μ Moles of Tris (pH 7.9), 10 mMoles $MgCl_2$, 0.5 mMole EDTA, 50 mMoles KCl, 0.04 mMole each of UTP, CTP, and GTP, 0.1 μ Ci ^{14}C -ATP, and chromatin containing three to five μ g of DNA. After incubation at 37° C for 20 minutes, the reaction mixture was rapidly chilled to 4° C and 0.1 ml of

bovine serum albumin (BSA, 1 mg/ml) was added. The reaction was stopped by addition of an equal volume of cold 10% TCA. After 30 minutes the precipitates were collected on Millipore filters and processed as described above.

DNA was determined either by the diphenylamine reaction of Burton (1956) or by the indole reaction described by Ceriotti (1952, 1955), using calf thymus DNA or salmon sperm DNA as a standard. Protein was determined by the method of Lowry *et al.* (1951), using bovine serum albumin or calf thymus histone as a standard.

Extraction of Histones

Nuclei were extracted with 0.25 N HCl for 20 hours at 4° C and centrifuged at 8000 x g for 30 minutes. The nuclear pellets were re-extracted twice for 30 minutes and the supernatants pooled. Nine volumes of acetone were added to the combined HCl extracts and histones were precipitated at 4° C for 16 hours. The histones were then collected by centrifugation at 10,000 x g for 30 minutes, washed with 30 ml of ethyl ether, followed by centrifugation at 10,000 x g for 30 minutes and evaporated to dryness in a vacuum desiccator.

Metabolism of Nuclear Proteins

The synthesis of chromatin proteins was studied in neuroblastoma cells grown in medium with and without FCS and with FCS plus db cAMP. Growth medium was removed from the monolayers and replaced with leucine- or tryptophan-free MEM containing 5 μ Ci/ml of L-leucine-[4,5-³H] (46 Ci/mM, New England Nuclear) or L-tryptophan-³H (2.7 Ci/mM, New England Nuclear), respectively. The cells were incubated in the presence of the labelled amino acid for 60 minutes prior to washing and harvesting.

To study acetylation of chromatin proteins, growth medium was

removed and replaced with MEM containing 50 to 100 $\mu\text{Ci/ml}$ of sodium acetate- ^3H (0.74 Ci/mM, New England Nuclear) for 60 minutes prior to harvesting.

To study phosphorylation of chromatin proteins, cells were incubated in phosphate-free MEM containing 60 to 100 $\mu\text{Ci/ml}$ of ^{32}P as $\text{H}_3^{32}\text{PO}_4$ for 60 minutes.

Histone synthesis and phosphorylation were examined in cells which were incubated in leucine- and phosphate-free MEM containing 5 $\mu\text{Ci/ml}$ leucine- ^3H and 60 to 100 $\mu\text{Ci/ml}$ of ^{32}P for 60 minutes.

Chromatin was prepared and histones extracted as described above and the proteins fractionated on polyacrylamide gels which were processed as described in the following section.

Polyacrylamide Gel Electrophoretic Fractionation of Chromatin Proteins.

Total chromatin proteins. Two SDS-polyacrylamide gel systems were used to analyze the molecular weight profile of total chromatin proteins: that of Ehorjee and Pederson (1972) using phosphate buffer and that of Laemmli (1970) using Tris-glycine buffer. Chromatin was prepared from cells from three Petri dishes or Blake flasks and was dissociated in 1.5 ml of the appropriate sample buffer (see below) in a Dounce homogenizer fitted with a Type "A" glass pestle. The sample was dialyzed overnight against 1000 volumes of the buffer at 22° C and sucrose was added to a final concentration of 15% (wt/vol). Samples were heated in a boiling water bath for two minutes before being applied to polyacrylamide gels prepared in glass tubes which had been treated with 1% Photoflo (vol/vol, Kodak).

Gels were prepared using acrylamide, N,N'-methylene-bisacrylamide (bis), and N,N,N',N'-tetramethylethylenediamine (TEMED) from Eastman

Kodak Company. Ammonium persulfate was obtained from Aldrich Chemicals and 2-mercaptoethanol from Matheson, Coleman and Bell.

Gels prepared according to the method of Bhargjee and Pederson were 7.5 cm x 0.6 cm and contained 7.5% acrylamide (wt/vol), 0.25% bis (wt/vol), 0.1% SDS (wt/vol), 0.1 M phosphate (pH 7.0), 0.5 M urea, 0.005 M EDTA, 0.05% TEMED (vol/vol), and 0.1% ammonium persulfate (wt/vol). The separating gel was covered with 0.1% SDS (wt/vol), 0.005% EDTA (wt/vol), and 0.1% ammonium persulfate (wt/vol) and allowed to polymerize at room temperature for 45 minutes. A one cm stacking gel was prepared with final concentrations of 2.5% acrylamide (wt/vol), 0.09% bis (wt/vol), 0.1% SDS (wt/vol), 0.01 M sodium phosphate (pH 6.0), 0.5 M urea, 0.005 M EDTA, 0.07% TEMED (vol/vol), and 0.08% ammonium persulfate (wt/vol). Samples were suspended in 0.1% SDS (wt/vol), 0.1% 2-mercaptoethanol (vol/vol), 0.01 M sodium phosphate (pH 7.0), and 15% sucrose (wt/vol). Bromophenol blue in the sample buffer was used as a dye marker. Samples were electrophoresed for six to seven hours with 8 mA/gel in a running buffer of 0.1% SDS (wt/vol), 0.1 M sodium phosphate (pH 7.0), and 0.005 M EDTA at room temperature.

Laemmli gels were 10 cm x 0.6 cm and contained 8% acrylamide (wt/vol), 0.2% bis (wt/vol), 0.1% SDS (wt/vol), 0.375 M Tris HCl (pH 8.8), 0.025% TEMED (vol/vol), and 0.025% ammonium persulfate (wt/vol). The stacking gel consisted of 0.25 ml of 3% acrylamide (wt/vol), 0.08% bis (wt/vol), 0.1% SDS (wt/vol), 0.125 M Tris HCl (pH 8.8), 0.025% TEMED (vol/vol) and 0.025% ammonium persulfate (wt/vol). Samples were suspended in 0.0625 M Tris HCl (pH 6.8), 2% SDS (wt/vol), 5% 2-mercaptoethanol (vol/vol), and 15% sucrose (wt/vol). Samples were electrophoresed for five hours at 2 mA/gel in a running buffer of 0.025 M Tris HCl (pH 8.3), 0.192 M glycine and 0.1% SDS (wt/vol).

Following completion of electrophoresis, gels were immediately removed from glass tubes and fixed in 12% TCA (wt/vol) in 40% ethanol (vol/vol) and 7% acetic acid (vol/vol) at room temperature for 12 hours. Gels were then washed with 40% ethanol--7% acetic acid and stained for five hours at 37° C with 0.25% Coomassie brilliant blue R (wt/vol, Sigma Chemicals) in 40% ethanol--7% acetic acid. Gels were destained electrophoretically in a Canalco Quick Gel Destainer or diffusion destained with 10% ethanol--7% acetic acid. Gels were stored in 7% acetic acid. The linear migration of proteins in proportion to the log molecular weight in both types of gels was verified using solutions of proteins of known molecular weight obtained from Sigma Chemicals and Pharmacia.

Histones. Histone polypeptides were fractionated electrophoretically according to charge and molecular weight on polyacrylamide gels prepared with acetic acid and urea according to the method of Panyim and Chalkley (1969). Gels contained 15% acrylamide (wt/vol), 0.5% bis (wt/vol), 2.5 M urea, 0.5% TEMED (vol/vol), 0.125% ammonium persulfate (wt/vol) and , 5.4% acetic acid (vol/vol). Gels were 9.0 cm x 0.6 cm and were pre-electrophoresed for four to five hours at 2 mA/gel. Samples, in 0.9 M acetic acid with 15% sucrose (wt/vol), were run for four hours at 2 mA/gel. Gels were fixed and stained simultaneously in 0.1% Amido black (wt/vol) in 20% ethanol (vol/vol)--7% acetic acid (vol/vol) for 12 hours and destained electrophoretically for 15 minutes.

Gels were scanned in a Beckman Acta II spectrophotometer at 590 nm for the SDS-polyacrylamide gels and at 620 nm for the acetic acid-urea polyacrylamide gels. The areas under the optical density profiles were integrated with a compensating polar planimeter (Keuffel and Esser

Company) to determine the amounts of protein in discrete molecular weight regions of the gels. Comparisons of the density of the Coomassie blue staining pattern were made on gels containing different amounts of protein to verify that the relationship between the protein concentration and the amount of dye binding was linear in the ranges of protein concentration commonly used.

For analysis of radioactive isotope incorporation into the various polypeptide bands, gels were frozen on dry ice and sliced into 1 mm fractions with a Hoeffler gel slicer. Slices were solubilized in 200 μ l of 35% hydrogen peroxide overnight at 37° C and then counted in a Triton X-100--toluene--Liquifluor cocktail (1:2:0.13) in a Beckman liquid scintillation counter.

Gels were photographed using a strong yellow VII filter (Kodak G15) and Kodak Ektapan film.

DNA Synthesis in Mouse Neuroblastoma Cells.

To study DNA synthesis, cells were inoculated into 150 mm plastic Petri dishes and the incorporation of thymidine-methyl- ^3H into TCA-insoluble material was determined. After 24 hours in MEM with 10% FCS, medium was changed to MEM plus fresh 10% FCS, MEM without FCS, or to MEM plus 10% FCS and 1.0 mM db cAMP. Cells were incubated for 24 hours and then incubated for 60 minutes with ^3H -thymidine (final concentration 1 $\mu\text{Ci/ml}$; specific activity 55 ci/mMol , Nuclear Dynamics). Cells were then mechanically harvested in cold balanced salt solution. Aliquots were taken to determine cell number, incorporation of ^3H -thymidine into TCA-soluble and -insoluble components of whole cells and of isolated nuclei, and amount of DNA in the various fractions. DNA was determined by the diphenylamine reaction of Burton (1956) using paraldehyde in the

diphenylamine reagent (Richards, 1974) and calf thymus DNA as a standard.

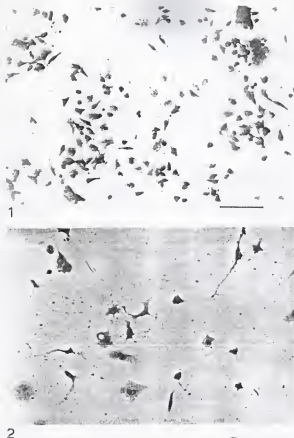
CHAPTER III

RESULTS

Cell Growth and Differentiation

Morphology

The morphology of living and fixed cultures of mouse neuroblastoma cells was investigated with the aid of phase contrast and bright field microscopy. Figures 1 through 7 represent cells grown under the various culture conditions. Cresyl violet is commonly used as a general cell and nuclear stain for nervous tissue. The Bodian silver protargol method is specific for neurons. A great deal of morphological diversity is seen in any one culture flask. In general, three populations of cells are present: small relatively regular round cells, flattened larger cells with processes and very large flattened cells with numerous vacuoles. Cells which had been maintained for 24 hours or longer without serum or in the presence of db cAMP tended to be larger than cells grown in serum-containing medium with numerous long branching processes. All cultures of neuroblastoma cells stained with silver stains and contained large amounts of argyrophillic material. Comparison of silver-stained neuroblastoma cells with human fibroblasts (Figure 8) revealed that the fibroblasts took up very little silver and only into the nuclei, while the nuclei of neuroblastoma cells stained intensely and the cytoplasm contained uniformly dispersed argyrophillic material. No evidence of fibrillar material in the cell bodies or processes of the neuroblastoma cells was visible or even resembled the fibers visible with the same stain in rat brain (Figure 9).



Figures 1 and 2. Mouse neuroblastoma cells from clone Neuro 2a fixed and stained with cresyl violet. Cells were grown in 25 cm² plastic flasks in MEM with 10% FCS for 24 hours. Medium was then changed to fresh MEM with 10% FCS (Figure 1) or to MEM without FCS (Figure 2) and cells were incubated for 24 hours. The bar represents 100 μ (magnification X 160).



Figures 3 and 4. Mouse neuroblastoma cells clone Neuro 2a grown in MEM plus 0.5 mM db cAMP (Figure 3) or 1.0 mM db cAMP for 24 hours. Cells were stained with cresyl violet. The bar represents 100 μ (magnification X 160).



5



6

Figures 5 and 6. Neuroblastoma cells grown on glass microscope slides and stained with Bodian silver protargol method (see Table 1). Following incubation for 24 hours in MEM with 10% FCS, cells were incubated for 24 hours in fresh MEM with 10% FCS (Figure 5) or MEM without serum (Figure 6). The bar represents 100 μ (magnification X 850).



Figure 7. As in Figures 5 and 6 but cells incubated in MEM plus 1.0 mM db cAMP.



Figure 8. Human diploid fibroblast ATCC 1121 grown for 48 hours in MEM with 10% FCS and stained with Bodian silver protargol method (magnification X 850).

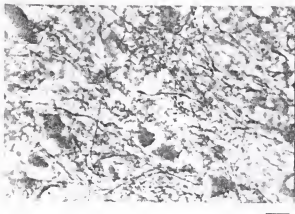


Figure 9. Rat brain section embedded in parafilm and stained and photographed as Figures 5 through 7. Note the fine neuronal processes and large neuronal cell bodies.

A comparison of the C1300 Neuro 2a neuroblastoma cells with a clone obtained from another laboratory was made in order to determine whether more recently cloned lines exhibit less morphological heterogeneity. Observations of hematoxylin and Giemsa-stained material (Figures 10-11), indicated that clone 2(D₁) consists of populations of cells similar to those seen in C1300 cultures; however, the large vacuolated cells are present in fewer numbers than in the Neuro 2a cell line.

Cell Number and Percent "Differentiation"

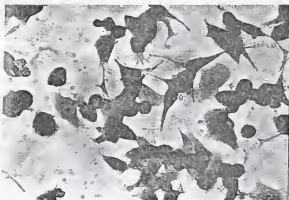
When mouse neuroblastoma C1300 cells were grown in medium containing 10% or 20% FCS, the majority of the cells remained round and relatively regular and cultures rapidly approached confluency. With either removal of serum or addition of db cAMP to the medium, cells became flattened, enlarged and developed one or more branching processes.

Figures 12 and 13 show the increase in what we have called "differentiation"--the presence of at least one process as long as the diameter of the cell body--over time following replacement of growth medium (MEM with either 10% or 20% FCS) with medium lacking serum or containing db cAMP. The shapes of the curves for "differentiation" in 10% and 20% FCS are almost identical for the first 24 hours. Both serum withdrawal and addition of db cAMP result in similar percentages of differentiated cells by 24 hours.

In Figure 14 the number of cells counted in each flask over time is indicated. Since the same fields were counted in each flask, these data reflect increases in cell number and density with time. Although the percent of cells with processes increases with time in the cultures grown in the presence of db cAMP, the cell number also continues to increase at the same rate as in the control cultures.



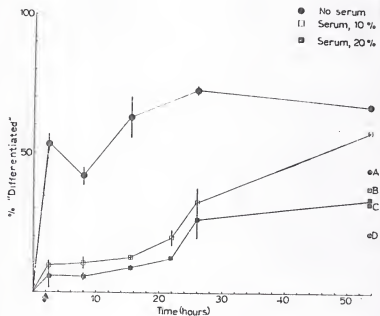
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11

Figures 10 and 11. Mouse neuroblastoma clone 2(D₁) grown in Ham's F12 medium and stained with Giemsa method. Magnification X 100 (Figure 10) and X 300 (Figure 11).

Figure 12. Differentiation of neuroblastoma cells. Small plastic tissue culture flasks were inoculated with cells in MEM plus 10% FCS at time 0. At 2 hours (arrow) medium was changed. Labelled points at 54 hours represent percent differentiation in flasks whose medium was replaced at 26 hours as follows: (A) MEM with no serum replaced with MEM plus 10% FCS; (B) MEM plus 10% FCS replaced with fresh MEM plus 10% FCS; (C) MEM plus 20% FCS replaced with fresh MEM plus 20% FCS; and (D) MEM with no serum replaced with MEM plus 20% FCS. Vertical bars represent standard error of the mean for four flasks at each point out to 26 hours.



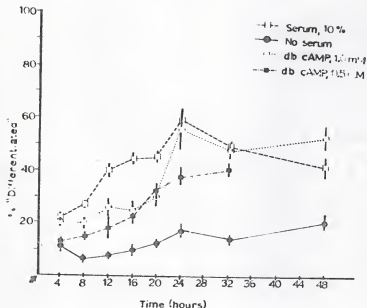


Figure 13. Differentiation of neuroblastoma cells. Cells were grown in MEM plus 10% FCS for four hours and then medium was changed (arrow). Vertical bars represent the SEM of counts from four flasks of cells.

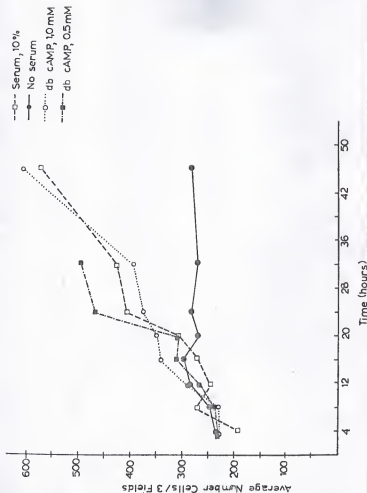


Figure 14. Cell number over time. The actual number of cells in each flask in the experiment summarized in Figure 13 is indicated. Points represent the average number of cells in three fields counted in four flasks.

When cultures grown in the absence of serum or in the presence of db cAMP for 24 hours were fed fresh medium containing serum (Figure 15), the level of "differentiation" decreased to control levels. In addition, when cultures which had been maintained in medium containing serum were re-fed, the level of differentiation decreased. Cultures exposed to medium without serum had many fewer cells and were visibly less dense than cultures grown with serum with or without db cAMP. No increase in the number of cells recoverable from the overlying medium was detected in cultures without serum.

Under all conditions, greater than 95% of the cells, both attached to the surface of the flask and recoverable from the overlying medium, were judged viable by dye exclusion.

Model of Growth

Using data consisting of cell counts for four populations of cells (10% FCS, no serum, 1.0 mM db cAMP, and 0.5 mM db cAMP) the growth of each if the four cell populations was modeled by a stationary birth and death process in collaboration with Rose Ray, Biostatistics Unit, Department of Statistics, University of Florida. Each population type is replicated four times; for each of the 16 flasks of cells, the cells in three fields were counted at 4, 8, 12, 16, 20, 24, 32 and 48 hours (48 hours is missing for the 0.5 mM db cAMP condition). At each time the number of non-differentiated (X) and the number of differentiated (Y) cells were counted.

The growth of each of the four cell types was modeled by the following birth and death process:

In any small amount of time dt the probability that a non-differentiated cell divides is λdt , the probability that a non-differentiated cell differentiates is μdt . The parameter λ is the birth rate, the parameter μ is the differentiation rate. These parameters are considered to be constant

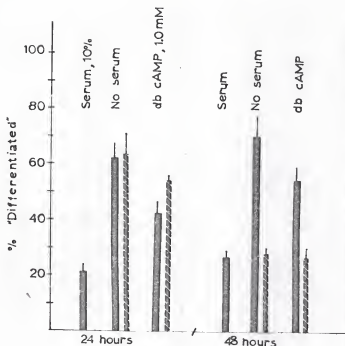


Figure 15. Reversibility of "differentiation." Cells were inoculated into 25 cm² flasks in MEM with 10% FCS. Medium was changed after 24 hours and the percent "differentiated" cells in each condition was determined. The medium in flasks represented by the solid bars was renewed and medium in flasks represented by striped bars was replaced with medium containing 10% FCS. At the end of 24 hours, percent "differentiation" was determined. Vertical lines represent the SEM of counts from four flasks.

throughout the 48 hours of the experiment. In addition, each cell is considered to behave independently of the others. The probability that more than one event (i.e., cell differentiation or cell division) occurs in a small amount of time dt is essentially zero. The differentiated cells do not divide and do not revert to the non-differentiated state.

This model contains only two unknown parameters, λ as the birth rate and μ the differentiation rate. These parameters were estimated by the method of least squares for each of the four populations; that is, for each of the four populations the sums

$$\sum [X_i - \hat{X}_i(\lambda, \mu)]^2$$

$$\sum [Y_i - \hat{Y}_i(\lambda, \mu)]^2$$

were minimized. Here $\hat{X}_i(\lambda, \mu)$ is the expected number of non-differentiated cells for this model if λ, μ are the parameters; similarly, $\hat{Y}_i(\lambda, \mu)$ is the expected number of differentiated cells if λ, μ are the parameters. These estimators are unbiased and normally distributed for large samples.

It was found that more accurate comparisons of the four populations could be made with a slight reparameterization of the model. The actual parameters estimated were $b = \lambda - \mu$ and μ . The parameter b can be described as the growth rate for the non-differentiated population of cells, i.e., the X population is increasing at rate λ and decreasing at rate μ ; $b = \lambda - \mu$ is the net growth rate.

The estimates for the four populations are as follows:

| Population | $\hat{b} = (\hat{\lambda} - \hat{\mu})$ | Variance | $\hat{\mu}$ | Variance |
|------------------|---|-----------------------|-------------|-----------------------|
| 1 10% FCS | .029 | 3.68×10^{-6} | .007 | 2.07×10^{-5} |
| 2 No serum | -.007 | 6.58×10^{-6} | .021 | 1.05×10^{-4} |
| 3 1.0 mM db cAMP | .009 | 2.32×10^{-6} | .028 | 6.04×10^{-5} |
| 4 0.5 mM db cAMP | .012 | 3.61×10^{-6} | .025 | 6.36×10^{-5} |

Students t-test was used to compare the four populations.

A total of 12 comparisons were made. There is strong evidence that populations 1, 2 and 3 have different growth parameters, $b = \lambda - \mu$. Populations 3 and 4 appear to have the same growth rate. There is no strong evidence for any population differences with respect to the differentiation rate μ . Population 1 appears to have a lower differentiation rate than the other three populations. The comparisons are as follows:

Comparisons for $b = \lambda - \mu$ (growth rate)

| | t | p |
|------------------|-------|--------|
| pop. 1 vs pop. 2 | 11.45 | .00005 |
| pop. 1 vs pop. 3 | 8.49 | .00005 |
| pop. 1 vs pop. 4 | 6.33 | .00005 |
| pop. 2 vs pop. 3 | -5.33 | .00005 |
| pop. 2 vs pop. 4 | -6.14 | .00005 |
| pop. 3 vs pop. 4 | -1.52 | .2000 |

Comparisons for μ (differentiation rate)

| | t | p |
|------------------|-------|-----|
| pop. 1 vs pop. 2 | -1.24 | NS |
| pop. 1 vs pop. 3 | -2.32 | .05 |
| pop. 1 vs pop. 4 | -1.92 | .20 |
| pop. 2 vs pop. 3 | -.54 | NS |
| pop. 2 vs pop. 4 | -.90 | NS |
| pop. 3 vs pop. 4 | .29 | NS |

The time invariant birth and death model was found to give only a mediocre fit to the data. There appears to be some change in the growth rate at approximately 20 hours.

Isolation of Nuclei and Chromatin

Examination of the pelleted material obtained after lysis of cells in NaCl-EDTA-Triton X-100 was carried out with both phase contrast and electron microscopy. Typical regions of the pellets are shown in Figures 16 and 17. Electron microscopic examination indicates that the pellet is composed primarily of intact nuclei without nuclear membrane components. The nuclear material, the classic chromatin of the microscopists, is

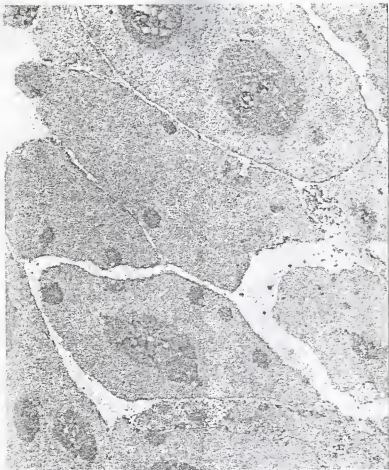


Figure 16. Electron micrograph of material pelleted after lysis of cells as described in the text. Note the prominent nucleoli and lack of nuclear membrane components. Magnification approximately X 15,000.



Figure 17. Higher power view of pelleted material shown in Figure 16. Note the lack of nuclear membrane material. Magnification approximately $\times 45,000$.

uniformly dispersed and nuclei contain one or more prominent nucleoli. Non-nuclear material is present to some degree; however, no well-defined cellular organelles other than nuclei are identifiable.

Following the mixing of ^3H -leucine-containing cytoplasmic fractions with unlabelled nuclear fractions, less than 1% of the tritium was recovered in the final chromatin preparation.

| | A ^3H -cpm cytoplasmic fraction | B ^3H -cpm final chromatin | B/A |
|-------------------------------------|--|---|-------|
| From cells grown with 10% FCS | 104,276 | 617 | 0.006 |
| From cells grown with no FCS | 72,407 | 233 | 0.003 |

Several attempts were made to characterize the protein and DNA content of the chromatin prepared from cells grown with and without 10% FCS. DNA and protein determinations were carried out directly on the chromatin pellet following lysis of nuclei in water and on fractions of the pellet following extraction of DNA and histones. Values of the DNA to protein ratio ranged from 1:0.25 to 1:2.66 for chromatin from cells grown in serum and from 1:2.17 to 1:6.96 for chromatin from cells grown without serum. Since very small volumes of material were available for each determination, the wide variation in results may result from difficulties in accurately pipetting very small volumes of extremely viscous material.

When histone to non-histone chromosomal protein ratios in chromatin from cells grown in different media were estimated from the areas under the optical density profiles of SDS-polyacrylamide gels (as described below), values ranged from 1.12 to 1.56 (see Table 5). Each set of gels

TABLE 5

Relative amounts of protein from intact nuclei of neuroblastoma cells electrophoretically fractionated on Ehorjee and Pederson SDS-polyacrylamide gels as described in the text.

| Sample | Fraction and MW X 10 ⁻³ | | | | | | Histone/ Nonhistone |
|------------------------------------|------------------------------------|-----------|-----------|-----------|-----------|-----------|------------------------|
| | A-B | C-E | F-J | K | I-O | P-R | |
| | 98.2-145.0 | 64.2-98.2 | 38.4-64.2 | 31.5-38.4 | 17.9-31.5 | 10.0-17.9 | |
| 1. Nuclei--with serum with TPCX | 32.9 | 31.6 | 25.1 | (32.8) | 10.4 | (67.2) | .94 |
| 2. (re-scan) | 33.7 | 32.0 | 25.6 | (32.6) | 8.6 | (67.5) | .98 |
| 3. | 32.8 | 30.2 | 24.4 | 30.5 | 12.4 | 69.5 | .79 |
| 4. with serum without TPCX | 33.9 | 29.4 | 24.8 | (27.2) | 11.9 | (72.8) | 1.04 |
| 5. | 31.8 | 28.9 | 25.8 | (27.5) | 13.3 | (72.4) | .94 |
| 6. without serum with TPCX | 27.5 | 30.5 | 26.5 | (29.5) | 15.4 | (70.5) | 1.04 |
| 7. without serum without TPCX | 30.5 | 29.5 | 26.3 | 26.8 | 13.7 | 73.2 | 1.09 |
| 8. | 34.2 | 31.5 | 25.5 | (30.9) | 8.8 | (69.1) | .98 |

Relative amounts of protein were determined by integrating the areas under the regions of the optical density profiles of the gels, scanned at 590 nm. Values for regions A-J and I-O are expressed as percent total non-histone protein and for K and P-R as percent total histone. Values in ()'s are uncertain since the optical density of the histone bands exceeded the upper detection limits for the spectrophotometer. Each line of data represents one gel.

yielded slightly different values; however the histone to non-histone ratio was always slightly greater for material from "differentiated" cultures. This variation reflects differences in background staining of the gels and limitations of defining limits of each band of polypeptides.

Comparison of gel profiles of polypeptides from intact nuclei and from chromatin does not reveal any clear differences in polypeptide band patterns as seen in the scans represented in Figures 18 and 19. When DNA determinations were made on the various supernatant and precipitate fractions obtained during the preparation of chromatin, DNA was only found in the isolated nuclear pellets and in the chromatin. The final supernatant following lysis of nuclei in distilled water did not contain any DNA.

When comparisons were made between nuclear and chromatin material prepared in the presence or absence of TPCK, a serine protease inhibitor, no differences were seen (Figure 20). Visual inspection of the gels revealed an apparent quantitative difference in one band; however analysis of the optical density scans and comparisons of the areas under various peaks did not reveal any consistent differences (Figure 20 and Tables 5 and 6). No difference in the recovery of histones prepared in the presence or absence of TPCK was noted.

Transcriptional Activity of Chromatin

The transcriptional activity of the material prepared from cells grown with or without serum or with serum and db cAMP was determined by assaying the ability of isolated chromatin to serve as a template for DNA-dependent RNA synthesis in a cell-free system. The incorporation of ^{14}C -ATP by E. coli RNA polymerase into acid-insoluble material was measured under three assay conditions in which the amount of DNA template

TABLE 6

Relative amounts of protein from chromatin of neuroblastoma cells electrophoretically fractionated on Bhoree and Pederson SDS-polyacrylamide gels as described in the text.

| Sample | Fraction and MW X 10 ⁻³ | | | | | | Histone/ Nonhistone |
|---------------------------------------|------------------------------------|-----------|-----------|-----------|-----------|-----------|------------------------|
| | A-B | C-E | F-J | K | L-O | P-R | |
| | 98.2-145.0 | 64.2-98.2 | 38.4-64.2 | 31.5-38.4 | 17.9-31.5 | 10.0-17.9 | |
| 1. Chromatin--with serum with TPCK | 37.5 | 27.8 | 18.9 | | 15.9 | | (1.39) |
| 2. | 36.4 | 26.3 | 21.2 | | 15.8 | | |
| 3. with serum without TPCK | 33.4 | 27.9 | 23.4 | | 15.2 | | |
| 4. | 34.4 | 25.4 | 22.3 | | 17.9 | | |
| 5. without serum with TPCK | 31.7 | 24.4 | 22.4 | | 21.5 | | (1.56) |
| 6. without serum without TPCK | 36.3 | 27.8 | 22.2 | | 13.8 | | |

To be continued/

Table 6 (Continued)

| Sample | Fraction and $MW \times 10^{-3}$ | | | | | | Histone/ Nonhistone |
|---------------------------------------|----------------------------------|-----------|-----------|-----------|-----------|--------|------------------------|
| | A-B | C-E | F-J | K | L-O | P-R | |
| 98.2-145.0 | 64.2-98.2 | 38.4-64.2 | 31.5-38.4 | 17.9-31.5 | 10.0-17.9 | | |
| 1. Chromatin--with serum with TPCK | 36.8 | 26.0 | 27.0 | (22.9) | 10.3 | (77.1) | 1.12 |
| 2. with serum without TPCK | 43.5 | 25.0 | 21.0 | (14.4) | 10.6 | (85.6) | 1.39 |
| 3. without serum with TPCK | 35.6 | 26.9 | 28.1 | (18.8) | 9.5 | (81.2) | 1.31 |
| 4. without serum without TPCK | 36.1 | 29.1 | 25.8 | (20.6) | 9.0 | (79.4) | 1.56 |

Relative amounts of protein were determined by integrating the areas under the regions of the optical density profiles of the gels, scanned at 590 nm. Values for regions A-J and L-O are expressed as percent total non-histone proteins and for K and P-R as percent total histone. Values in (%) are uncertain since the optical density of the histone bands exceeded the upper detection limits for the spectrophotometer. Each line of data represents one gel.

Data sets A and B represent two different experiments and sets of gels.

Figure 18. Comparison of pattern of Coomassie blue staining of proteins fractionated electrophoretically on Bhargava and Pederson gels from intact nuclei of mouse neuroblastoma cells grown in MEM with 10% FCS (Serum) or without FCS (No serum). Nuclei were isolated from cells in the presence of IFK.

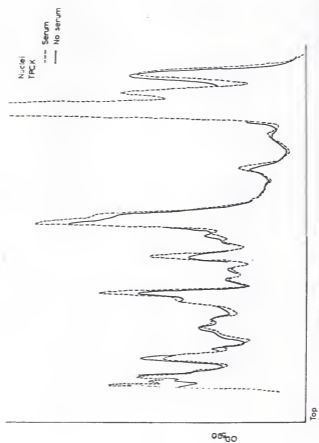


Figure 19. Comparison of the patterns of staining of proteins fractionated electrophoretically on Ehorjee and Pederson gels from chromatin prepared from isolated nuclei of neuroblastoma cells grown with (Serum) and without (No serum) FCS. Chromatin was isolated without the use of TPCK.

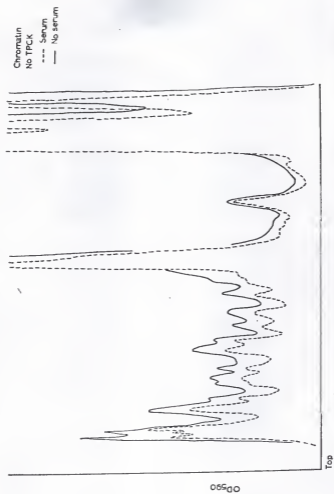
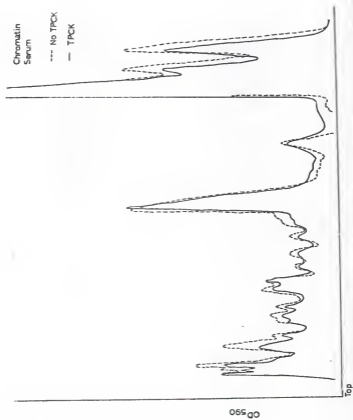


Figure 20. Comparison of the staining pattern of proteins fractionated electrophoretically on Bhorjee and Pederson gels from samples of chromatin prepared with and without TPCK from neuroblastoma cells grown in MEM with 10% FCS.



was rate limiting. The results of these assays are shown in Figure 21 and Tables 7 and 8. Under all conditions, template activity for chromatin from cells grown in the presence of serum was several-fold higher than that of chromatin from cells exposed to no serum conditions or to db cAMP.

Electrophoretic Fractionation of Nuclear and Chromatin Proteins

Total proteins from isolated nuclei and from lysed nuclei (chromatin) were investigated in two different SDS-polyacrylamide gel systems. The migration of proteins of known molecular weight in a linear fashion proportional to the log molecular weight is shown in Figures 22 and 23 for each type of gel. Histone H1 migrates in an anomalous fashion in the Bhorjee and Pederson gels (Hayashi *et al.*, 1974) and appears in the region denoted by K in Figure 27. Figures 24 and 25 show the Bhorjee and Pederson gel patterns of material solubilized from isolated nuclei and from chromatin prepared in the presence of and in the absence of TPCK.

When different amounts of protein were applied to a series of gels, the areas under the optical density profiles corresponded linearly to the protein concentration applied within the range of 5 to 30 μ g of protein as determined by the method of Lowry *et al.* (1951).

Tables 5 and 6 indicate the distribution of protein, expressed as percent of the total, in different regions of the gels. No consistent differences appear between intact nuclei and chromatin, or chromatin prepared with and without TPCK. Variability from one set of gels to another (Table 5 and Table 6, A and B each represent a different set of gels), as well as from one scan to another of the same gel (Table 5, 1 and 2) is apparent. Although this method of analysis has a limited degree of resolution, we chose to apply it to an investigation of the

Figure 21. The ability of chromatin from isolated nuclei of mouse neuroblastoma cells to support the incorporation of ^{14}C -ATP into acid-insoluble material by *E. coli* RNA polymerase was determined as described in the text. The actual data are presented in Table 7. Chromatin was prepared from cells which had been grown in MEM with 10% FCS (Serum), MEM without FCS (No serum), and in MEM with 10% FCS plus 1.0 mM dbcAMP (db cAMP). Incorporation of ATP using naked DNA as a template was considered to represent 100% template capacity. Preparations of chromatin and assay mixture minus RNA polymerase (No enzyme) revealed little endogenous activity.

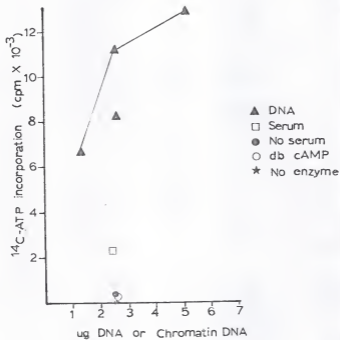


TABLE 7

Transcription assay using Burgess fraction IV E. coli RNA polymerase. Incorporation of ^{14}C -ATP into acid-insoluble material with either DNA or chromatin as a template.

| <u>Template material</u> | | ^{14}C -ATP cpm |
|---|--|--------------------------|
| | | <u>With enzyme</u> |
| I <u>Murphy et al. assay conditions</u> | | |
| Calf thymus DNA | | |
| 0 μg | | 127 |
| 5.5 | | 1554 |
| 11 | | 1698 |
| 22 | | 1651 |
| 55 | | 1605 |
| 77 | | 1790 |
| 100 | | 1854 |
| Chromatin from cells with serum | | |
| 1.0 μg DNA | | 671 |
| 2.0 | | 1179 |
| Chromatin from cells without serum | | |
| 0.6 μg DNA | | 245 |
| 1.2 | | 294 |
| II <u>Repeat with new batch enzyme</u> | | |
| Chromatin from cells with serum | | |
| 2.0 μg DNA | | 1537 |
| 4.0 | | 3612 |
| Chromatin from cells without serum | | |
| 3.0 μg DNA | | 440 |
| 12.0 | | 409 |
| III <u>Bonner et al. assay conditions</u> | | |
| Chromatin from cells with serum | | |
| 0 μg DNA | | 1601 |
| 6.0 | | 4376 |
| 12.0 | | 6768 |
| 30.0 | | 10867 |
| 60.0 | | 14899 |
| 108.0 | | 14335 |
| Chromatin from cells without serum | | |
| 2.4 μg DNA | | 3608 |
| 4.8 | | 3122 |
| 12.0 | | 4140 |
| 24.0 | | 6721 |
| 43.2 | | 11393 |

TABLE 8

Transcription assay using Burgess fraction IV *E. coli* RNA polymerase. Incorporation of ^{14}C -ATP into acid-insoluble material with either DNA or chromatin as a template and a modification of Bonner *et al.* assay conditions.

| Template material | ^{14}C -ATP | | % Template Transcribed |
|--|-------------------------|---------------------|------------------------|
| | Without enzyme | With enzyme | |
| Salmon sperm DNA | | | |
| 0 μg | | 228 | |
| 1.28 | | 6627 | |
| 2.56 | | 11072 | |
| 5.12 | | 12697 | |
| 10.24 | | 24197 | |
| 2.56 | $261 \pm 28.7(2)^{a,b}$ | $8732 \pm 757.4(2)$ | 100.0 |
| Chromatin from cells with serum | | | |
| 2.34 μg DNA | $60 \pm 0.2(2)$ | $2311 \pm 30.1(4)$ | 26.5 |
| Chromatin from cell without serum | | | |
| 2.43 μg DNA | $178 \pm 9.3(2)$ | $232 \pm 1.4(4)$ | 2.7 |
| Chromatin from cells with serum plus db cAMP | | | |
| 2.38 μg DNA | $110 \pm 6.5(2)$ | $222 \pm 19.0(4)$ | 2.5 |

^a % Template transcribed calculated using cpm incorporated with naked DNA as 100%.

^b Numbers in parentheses represent numbers of samples. Data represent mean \pm SEM.

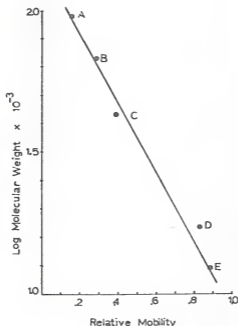


Figure 22. Migration of proteins of known molecular weight in 7.5% polyacrylamide gels prepared according to the method of Bhoree and Pederson as described in the text. Ten micrograms of protein were applied to the gels in the sample buffer with a final concentration of 1 $\mu\text{g}/\text{ml}$: (A) phosphorylase A, 94,000; (B) bovine serum albumin, 68,000; (C) ovalbumin, 43,000; (D) horse myoglobin, 17,200; (E) cytochrome C, 12,380.

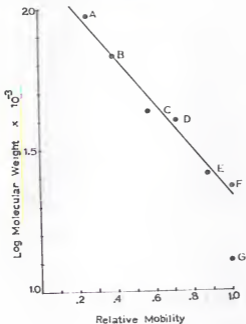


Figure 23. Migration of proteins of known molecular weight in 8% polyacrylamide gel prepared according to the method of Laemmli as described in the text. Approximately 10 μ g of protein were applied to each gel. Gels containing mixtures of the proteins were also run: (A) phosphorylase A, 94,000; (B) bovine serum albumin, 68,000; (C) ovalbumin, 43,000; (D) aldolase, 40,000; (E) chymotrypsinogen, 25,700; (F) trypsin, 23,300; (G) RNase, 13,700.

Figure 24. Total nuclear proteins on SDS-acrylamide gels prepared according to the method of Bhorjee and Pederson. Gels were stained with Coomassie brilliant blue. From left to right, material prepared from total nuclei of cells grown in the presence of 10% FCS isolated with (a) and without TPCK (b), and material prepared from total nuclei of cells grown in serum-free MEM prepared with (c) and without TPCK (d).

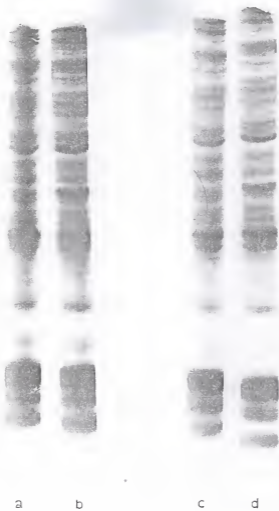


Figure 25. Bhorjee and Pederson gel patterns of chromatin prepared from nuclei isolated from cells grown in the presence of serum and isolated with TPCK (a) and without TPCK (b) and chromatin prepared from nuclei isolated from cells grown in serum-free MEM and prepared in the presence (c) and absence of TPCK (d).



composition and metabolism of the chromatin proteins from neuroblastoma cells grown with and without serum and with serum plus db cAMP.

Laemmli gels were run of chromatin samples in an attempt to separate the high molecular weight bands to a greater degree than was possible with the Bhorjee and Pederson gels. This gel system separates high molecular weight polypeptides slightly better than the Bhorjee and Pederson gels; however, proteins of less than 25,000 molecular weight migrate with the buffer front (see Figure 23). No clearly definable differences were detectable among the chromatin samples from cells grown in serum, no serum, or serum and db cAMP.

All five major classes of histone fractions are clearly visible on acetic acid-urea polyacrylamide gels prepared according to the method of Panyim and Chalkley (Figure 26). The electrophoretic mobilities of the histone fractions do not differ significantly as a function of the medium in which the cells were grown. The relative amounts of histone protein present in each of the five histone fractions is also similar (Table 9). No attempt was made to compare the acid-extractable non-histone polypeptides which migrate more slowly in the acetic-acid urea gels than do the histones.

Metabolism of Total Chromatin Polypeptides

To study the composition and synthesis of the non-histone chromatin proteins from neuroblastoma cells grown with and without serum and with db cAMP, cells were labelled with either ^3H -leucine or ^3H -tryptophan and the proteins from isolated chromatin were fractionated electrophoretically on Bhorjee and Pederson gels. Since histones do not contain tryptophan residues, incorporation of radioactivity is thought to reflect non-histone chromosomal protein synthesis. Figure 27 illustrates the electrophoretic profiles of total chromosomal polypeptides and the incorporation

Figure 26. Electrophoretic fractionation of histones from nuclei of cells grown in the presence of 10% FCS (a), in serum-free medium (b), and in medium with serum and 1.0 mM db cAMP (c) on acetic acid-urea-polyacrylamide gels prepared according to the method of Panyin and Chalkley as described in the text.

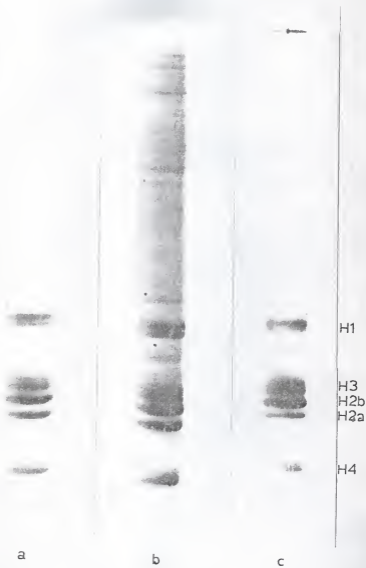


TABLE 9

Relative amounts of histone fractions in nuclei from neuroblastoma cells grown with and without serum and with serum plus db cAMP.

| | <u>Histone Fractions</u> | | | | | | | | |
|---|--------------------------|------|-----|----------------|------|-----|------|------|-----|
| | H1 | | | H3 + H2B + H2A | | | H4 | | |
| Nuclei from cells grown with serum | .18, | .16, | .19 | .65, | .67, | .68 | .18, | .18, | .15 |
| Nuclei from cells grown without serum | .17, | .17, | .19 | .63, | .66, | .68 | .21, | .18, | .15 |
| Nuclei from cells grown with 1.0 mM db cAMP | .21, | .17, | .18 | .60, | .66, | .69 | .20, | .18, | .13 |

Gels were stained with Amido Black, destained electrophoretically and scanned at 620 nm. Relative amounts of protein in each histone fraction were determined by integrating areas under each peak of the gel scan and are expressed as a fraction of the total protein. Data represent values from three gels, one each from three different experiments.

of radioactivity into various molecular weight fractions of nonhistone chromosomal proteins. Peaks P, Q and R represent histone fractions H2B + H3, H2A, and H4 (for discussion of histone nomenclature see Bradbury, 1975). As mentioned previously, histone H1 migrates anomalously in the 37,000 molecular weight region of the gels present in region K (Hayashi *et al.*, 1974). The relative amounts of protein in each region of the gels are similar for chromatin prepared from cells grown with and without serum (Table 10). An analysis of Figure 27, A and B, indicates variations occur in specific fractions. Peak A, which consists of nonhistone chromosomal polypeptides that migrate in the 120,000 to 130,000 molecular weight region of these gels, is more pronounced in chromatin from cells grown without serum or with db cAMP. Variations may also be evident in the E complex, which contains non-histone chromosomal polypeptides of molecular weight 64,000 to 78,000.

In the 120,000 to 145,000 molecular weight region of the gel, a two- to three-fold increase in the incorporation of ^3H -tryptophan is evident in the nonhistone polypeptides which are synthesized and associated with the chromatin of cells grown in the presence of serum as compared to similar material from cells grown without serum. A corresponding increase in the specific activity of these polypeptides is indicated in Table 10. There is an apparent increase in the specific activity of peak K in chromatin from cells grown in the presence of serum; however, the presence of H1 histone in this region makes it impossible to assign a specific activity to the nonhistone chromosomal protein component.

In another experiment, the incorporation of ^3H -tryptophan into chromatin components of cells grown with 1.0 mM db cAMP and 10% FCS was

Figure 27. SDS-polyacrylamide fractionation of chromatin proteins from nuclei of cells grown in the presence (a) and absence of serum (b). The incorporation of ^3H -tryptophan into each gel fraction is indicated by the dotted line and the solid line represents the optical density profile of the gels shown in Figure 25.

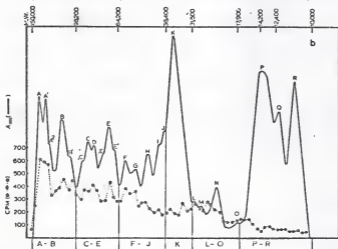
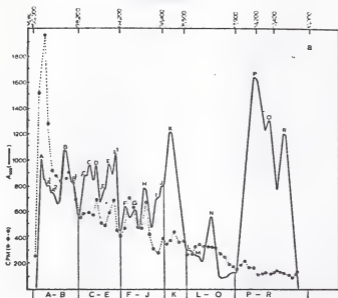


TABLE 10

Relative amounts of protein and incorporation [^3H] tryptophan in nonhistone chromosomal protein fractions of mouse neuroblastoma cells grown with and without serum.

| | Fraction and $\text{MW} \times 10^{-3}$ | | | | |
|-------------------|---|-----------|-----------|-----------|-----------|
| | A-B | C-E | F-J | K | L-O |
| Sample | 98.2-145.0 | 64.2-98.2 | 38.4-64.2 | 31.5-38.4 | 17.9-31.4 |
| With serum | | | | | |
| Relative amount | 18.17 | 18.13 | 14.64 | 10.26 | 6.78 |
| Specific activity | 16.2 | 7.3 | 8.0 | (4.3) | 8.6 |
| Without serum | | | | | |
| Relative amounts | 17.87 | 16.26 | 15.76 | 13.75 | 5.93 |
| Specific activity | 6.6 | 6.0 | 5.1 | (2.3) | 9.2 |

Total chromosomal proteins from cells labelled with [^3H] tryptophan were fractionated electrophoretically on Bhoree and Pederson polyacrylamide gels. The gels were stained with Coomassie Brilliant Blue, detected electrophoretically, and scanned at 600 nm. Relative amounts of protein in each fraction were determined by integrating the area under the scan and are expressed as percent of total protein. Specific activities of each molecular weight (MW) region were calculated by dividing total ^3H cpm by the area under that region of the scan. Histones migrate in regions K and P-R, and therefore specific activities for nonhistone proteins cannot be reliably assigned to these fractions.

determined. The optical density profiles (see Fig 30) are similar to those for chromatin from cells grown with and without serum. Figure 28 shows that a similar difference in incorporation of tryptophan into the nonhistone polypeptides which migrate in the high molecular weight regions of the gels exists as the difference seen for cells grown with and without serum. Consistent with the decreased incorporation of tryptophan into the proteins from cells in which "differentiation" has been induced by serum withdrawal, a similar decreased rate of synthesis of these polypeptides is apparent in cells in which "differentiation" is induced by db cAMP.

Post-translational modifications of chromosomal proteins, such as acetylation and phosphorylation, have been implicated in the regulation of gene expression. Such modifications of chromosomal polypeptides have been correlated with the activation of transcription in a broad spectrum of biological systems (Stein *et al.*, 1976). We therefore investigated the incorporation of ^3H -sodium acetate and ^{32}P into the various polypeptide components of chromatin from neuroblastoma cells.

Following 60 minutes of incubation with MEM containing ^3H -sodium acetate, chromatin was prepared from cells grown with and without the presence of serum and with serum plus 1.0 mM db cAMP. The chromosomal polypeptides were fractionated as described on Ehorjee and Pederson gels. In Fig 29 shows the pattern of incorporation of acetate into the various molecular weight regions of the gels. When the total number of counts recovered from each gel was compared with the number of counts applied, between 70 and 90% of the radioactivity was found to enter the gels. This was a similar level of recovery to that obtained using tritiated-amino acid incorporation. Under all growth conditions,

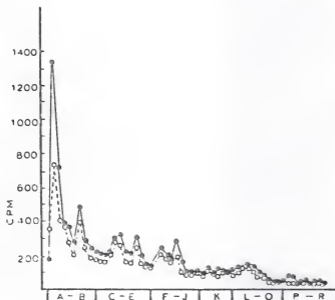
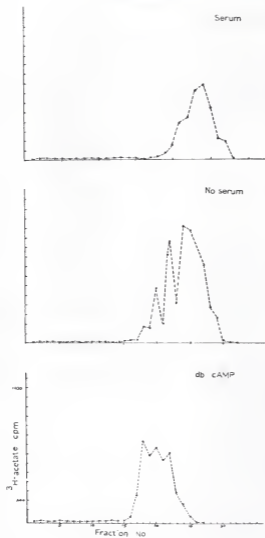


Figure 28. Incorporation of ^3H -tryptophan into chromatin proteins isolated from nuclei of cells grown in the presence of serum (solid line) and in the presence of serum and db cAMP (broken line). The optical density profiles of these gels are similar to the ones shown in Figure 27.

Figure 29. Pattern of incorporation of ^3H -acetate into chromatin proteins of cells grown with serum, with no serum, and with serum plus 1.0 mM db cAMP as described in the text. Optical density profiles of the staining patterns of the gels indicate that the fractions which contain greater than background amounts of ^3H -acetate are in the histone regions of each of the gels.



acetylation of nuclear polypeptides primarily involve the histones. Little, if any, label was recovered from nonhistone regions of the gels. Specific investigation of histone acetylation will be reported below.

Chromatin was prepared and fractionated electrophoretically following incubation of the cells in phosphate-free MEM containing ^{32}P . The incorporation of ^{32}P into the various regions of the Bhorjee and Pederson gels is shown in Figure 30. No significant difference in the phosphorylation pattern could be discerned. Significant incorporation of ^{32}P into the polypeptides which migrate in the higher molecular weight regions of the gels occurs in all three growth conditions. No preferential incorporation into the nuclear proteins of "differentiated" or proliferating mouse neuroblastoma cells appears to occur.

Metabolism of Histones

We then examined the metabolism of the histones which were extracted from isolated nuclei with dilute mineral acid. As mentioned above, no variation is seen in the distribution of the histone components extracted from nuclei of cells grown with or without serum or with db cAMP. When histones were extracted from nuclei of cells incubated in the presence of ^3H -leucine and fractionated electrophoretically on Panyin and Chalkley acetic-acid-urea polyacrylamide gels, the patterns seen in Figure 31 were obtained. An estimate of the relative specific activity of each gel region, computed from the amount of radioactivity and the area under each peak in the optical density scan, from three such experiments, is shown in Table 11. The experiment summarized in Table 11 indicates that the incorporation of leucine was greater in histones from cells grown in the presence of serum as compared to cells grown without serum or with db cAMP. However, caution should be exercised

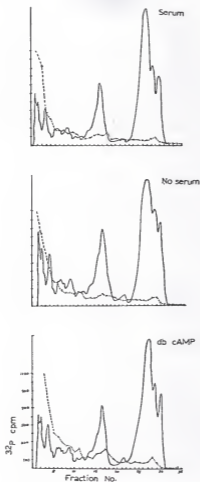


Figure 30. Incorporation of ^{32}P into chromatin proteins. The broken line represents the ^{32}P cpm and the solid line represents the optical density scan at 590 nm.

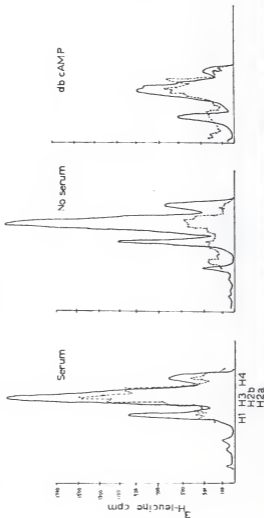


Figure 31. Pattern of incorporation of ^3H -leucine into histones extracted from isolated nuclei after incubating cells for 60 minutes in the presence of ^3H -leucine in leucine-free MEM as described in the text. Relative specific activities of the three main histone regions are shown in Table 11. The broken line represents ^3H -cpm and the solid line the optical density profile of Amido black staining pattern of the histone polypeptides. The top of the gels is to the left.

TABLE 11

Histone synthesis in neuroblastoma cells.

| Histone fraction | With serum | Without serum | With db cAMP |
|------------------|------------|---------------|--------------|
| Experiment A | | | |
| H1 | 22.2 | 18.8 | 12.6 |
| H3 | | | |
| H2b | 28.7 | 10.7 | 6.8 |
| H2a | | | |
| H4 | 20.2 | 6.8 | 19.2 |
| Experiment B | | | |
| H1 | 152.7 | 163.4 | |
| H3 | 58.3 | 100.4 | |
| H2b | 229.1 | 121.2 | |
| H2a | 75.5 | 603.0 | |
| H4 | 63.5 | 71.4 | |
| Experiment C | | | |
| H1 | 10.3 | 11.6 | 20.9 |
| H3 | | | |
| H2b | 3.8 | 4.7 | 5.4 |
| H2a | | | |
| H4 | 3.0 | 4.3 | 1.2 |

Histones were extracted from nuclei isolated from cells which had been incubated with ^3H -leucine. The data are estimates of relative specific activities of the various histone fractions as calculated by comparing the amount of radioactivity with the area under each peak of the optical density scan. Cells were incubated for 24 hours prior to labelling in MEM with 10% FCS, in MEM without serum, or in MEM with 10% FCS and 1.0 mM db cAMP.

in interpreting these data since results of two similar experiments shown in Table 11B and 11C do not show the same trend.

As determined from an analysis of total chromatin proteins on Bhorjee and Pederson gels, acetylation of nuclear proteins primarily involves the histone polypeptides. When histones were extracted from isolated nuclei of cells which had been incubated with ^3H -sodium acetate, no significant difference in the rate of acetate incorporation into the various histone fractions was observed from cells grown under the three growth conditions. As seen in Figure 32 and Table 12, sodium acetate incorporation was the greatest in the H3 and H4 fractions.

We investigated the incorporation of ^{32}P into the histone fractions of neuroblastoma cell chromatin, shown in Fig. 33. In all cases H1 was the most highly phosphorylated histone fraction (Table 13). No significant difference in phosphorylation pattern was detectable in two different experiments for the three growth conditions.

DNA Synthesis

A comparison of the degree of DNA synthesis in cultures of mouse neuroblastoma cells grown with the three media was made. The results are shown below. Little difference is detectable between the cells grown with and without serum. In cultures exposed to db cAMP, slightly lower levels of DNA synthesis occur than in the other conditions. The data represent the means and standard error of the mean of duplicate determinations on independent Petri dishes of cells. No differences were seen in acid-soluble counts.

| Source of nuclear sample | Acid-insoluble cmp ^3H -thymidine from isolated nuclei |
|--------------------------|--|
| Cells grown with serum | 225.9 \pm 4.8 |
| without serum | 264.7 \pm 49.6 |
| with serum + db cAMP | 162.4 \pm 16.5 |

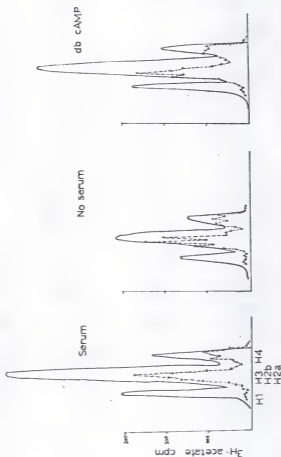


Figure 32. Pattern of incorporation of ^3H -acetate into the histone fractions. Relative specific activities of the three main histone regions are shown in Table 12. The broken line represents radioactivity and the solid line the optical density profile of the gel.

TABLE 12

Acetylation of the histone fractions of mouse neuroblastoma cells.

| | <u>Serum</u> | <u>No serum</u> | <u>db cAMP</u> |
|-----|--------------|-----------------|----------------|
| H1 | 0.8 | 1.0 | 1.4 |
| H3 | | | |
| H3b | 2.7 | 4.0 | 3.1 |
| H2a | | | |
| H4 | 4.6 | 5.1 | 5.6 |

Cells were incubated with ^3H -sodium acetate. Histones were extracted from isolated nuclei and electrophoretically fractionated as described in the text on urea-acetic acid-polyacrylamide gels. Values reported represent relative specific activities (^3H cpm/unit protein) determined from the 3H cpm in each region and amount of protein in each region of the gel scan as shown in Figure 22.

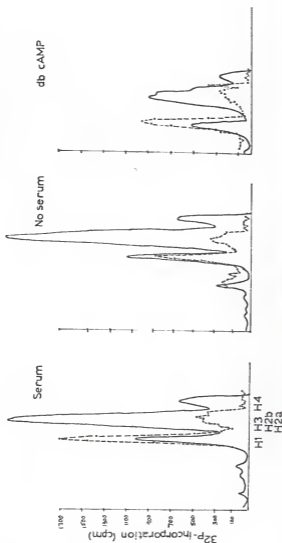


Figure 33. Pattern of incorporation of ^{32}P into histones following a 60-min pulse with ^{32}P in phosphate-free MM as described in the text. The broken line represents the radioactive content of each fraction and the solid line the optical density profile. Relative specific activities of each region are presented in Table 13.

TABLE 13

Phosphorylation of the various histone fractions of mouse neuroblastoma cells.

| Histone fraction | With serum | No serum | Serum + db cAMP |
|------------------|------------|----------|-----------------|
| Experiment 1 | | | |
| H1 | 308.8 | 444.1 | |
| H3 | 29.1 | 45.8 | |
| H2b | | | |
| H2a | 42.1 | 100.1 | |
| H4 | 15.0 | 22.5 | |
| Experiment 2 | | | |
| H1 | 64.5 | 32.3 | 89.5 |
| H3 | | | |
| H2b | 9.2 | 8.5 | 10.3 |
| H2a | | | |
| H4 | 3.2 | 3.4 | 4.1 |

Cells were incubated with $H_3^{32}PO_4$ in phosphate-free MEM as described in the text. Nuclei were isolated and histones extracted and run on polyacrylamide-urea-acetic acid gels. Values reported are relative specific activities calculated by dividing the ^{32}P cpm in each region of the gel by the units of protein determined by integrating the area under the optical density scan of the gel as shown in Figure 33 for Experiment 2.

CHAPTER IV

DISCUSSION

Morphology and Growth

The morphology and growth pattern of the mouse neuroblastoma C1300 cell line used in these studies are similar to those reported by numerous other laboratories. Ross et al. (1975) and Prasad (1975) stress the heterogeneity of the cells in both size and shape and the variability of the length, degree and pattern of branching of the processes. These processes have been designated "neurites," rather than axons since, as pointed out by Haffke and Seeds (1975), their function is not yet known. Numerous large vacuolated cells are present in the cultures under all growth conditions. These have been noted by Prasad (1971 a, b), Prasad and Sheppard (1972) and by Schubert et al. (1969) in cultures induced to "differentiate" with X-ray, bromodeoxyuridine, and in serum-free medium. Ross et al. (1975) noted that the cultures appeared to become more homogeneous as the cell density increased and the cells became confluent. This may reflect the inability of the observer to determine accurately the boundaries of individual cells as they crowd one another. Morphologic heterogeneity has been observed and described in even the most recently cloned cell lines and is thought to reflect both variations in the numbers of chromosomes present in the cells, even within a clone (Ciesielski-Treska et al., 1975), and microheterogeneities in the culture dish or flask surface (Schubert et al., 1973).

Numerous characteristics of the neuroblastoma cells have been

reported to change when the cells are exposed to serum-free medium or to medium supplemented with db cAMP. Amongst the histologic properties of the cells, the "differentiated" neuroblastoma cells have been reported to stain intensely with Bodian silver methods (Schubert et al., 1969, 1973) and Minna et al., 1971). Such argyrophilia has been correlated with early neuronal maturation and seems to be neuron-specific, as discussed in an earlier section of this paper. Our studies, however, indicate that the neuroblastoma cells under all three culture conditions stained intensely with Bodian silver protargol. Even the cells in the cultures grown with complete medium, conditions which promote cell division rather than "differentiation," stained intensely with silver. This supports the suggestion that the neuroblastoma cells do not represent an undifferentiated stem cell population when in the dividing state, but have already undergone the process of restriction to a neuron-like cell type. Therefore, the alteration from a population of cells predominantly composed of round dividing cells to one in which the majority of cells possess one or more long neurites represents the expression of a limited amount of genetic information, perhaps shared in common with neurons. It still reflects a major alteration in the expression of the cell's genetic information and occurs over a wide range of characteristics of the cells including ones which are primarily morphological, biochemical and electrophysiological.

It must be kept in mind, then, that the phenomenon which has been called "differentiation" by the investigators of the neuroblastoma cell line is an alteration in the phenotypic expression of cells which are apparently already determined to be neuron-like, although they continue to divide, into a population which overtly displays neuronal characteristics.

The proliferating cells do represent a stem cell population, but one which has a limited potential, much like the matrix cells of the wall of the early neural tube.

Cell Number and Differentiation

The time course of the phenomenon which we will continue to call "differentiation," in both serum-free medium and in medium containing db cAMP, is similar to that reported by several other investigators. Within several hours after changing the medium, more cells are found with processes than remain round. The more rapid change in the level of differentiation in cultures grown in the presence of 10% FCS compared with those grown in the presence of 20% FCS suggests that the quantity of serum factors necessary for the maintenance of a predominantly round cell population begins to drop below a threshold value by 24 hours in the 10% FCS cultures. The alterations induced by serum withdrawal and db cAMP seem to be completely reversible when cultures are re-fed with serum-containing medium after 24 hours. This contrasts with numerous reports by Prasad (see Prasad, 1975) that "differentiation" induced by exposure to db cAMP is irreversible. However, his cultures were exposed to db cAMP for three days or longer.

Cell number does not appear to be affected by db cAMP. Early studies reported that cell division was inhibited by db cAMP (as inferred from the lack of increase in cell number). It is now thought that this may be a result of contamination of early commercial supplies of db cAMP with butyric acid, a breakdown product of db cAMP which has been reported to prevent the increase in cell number by itself.

In cultures grown in serum-free medium, cell number reached a

plateau value early and ceased to increase over time. No increase in either dead or floating cells was noted in these cultures. Processes continued to elongate and became very spindly, with numerous branch points. It has been suggested that under conditions of no serum, cells are unable to attach or maintain their attachment. If this were the case, one might expect to find an increase in the number of floating cells. The problem of the role of adhesion of the cell to the surface and its ability to round up to divide is a controversial one. The fact that cells do not seem to alter their contact with the flask surface when exposed to db cAMP and are still induced to differentiate suggests that adhesion alone is insufficient to account for the phenomenon of "differentiation."

Our initial model assumed that cells which were differentiated could not divide; however, this model fitted the data only poorly. In order for cell numbers to increase simultaneously with percent differentiation, as occurs in the presence of both concentrations of db cAMP, either there must be a residual stem cell population which divides rapidly or all the cells in the population cycle between the division mode and the differentiated mode. Whether a decrease in the transit time of cells through the cell cycle occurs with db cAMP has not been determined.

An attempt to determine which cells continue to synthesize DNA under the three culture conditions was made using autoradiographic methods. However, the level of background of the autoradiograms, coupled with the geometry of a number of the cells, made it impossible to analyze and compare the slides adequately.

Isolation of Nuclei and Chromatin

Nuclei isolated by the methods used here yielded preparations which

were devoid of nuclear membranes. No anatomic barrier to diffusion into or out of the nuclei was present, therefore. The mixing experiment in which very little labelled amino acid-containing material was detected in the final chromatin material suggests that little binding to the isolated nuclei occurs following the first wash. However, we cannot rule out irreversible binding of cytoplasmic material prior to and during the first wash following detergent treatment. No attempt was made to determine the amount of material lost from the nuclei during the extraction procedure.

The variabilities in the protein:DNA ratios of the isolated chromatin material perhaps reflect the small volumes being pipetted. With very high viscosity, this could represent a significant source of error (Kelly, 1975). The histone to nonhistone ratios determined from an analysis of the areas under the optical density profiles are inherently inaccurate, especially because the histone regions were often too dense to be analyzed efficiently. The difference in values obtained across gels and in the scans from one gel to another probably represent small differences in the background staining of the gels, in the homogeneity of the gel and the limited sensitivity of the method. A linear relationship between the area integrated, which depends on the intensity of staining of the bands, and the amount of protein in each region exists within the range of zero to ten μg of protein as determined by us and by Elgin (1972) and Kelly (1975). We usually apply approximately 50 μg of protein per gel which is actually spread over a number of polypeptide bands and therefore each band should be within the range of linearity of the staining method (an individual protein need only contribute 0.3% (wt/wt) of a 50 μg heterogeneous sample to be resolved, Kelly, 1975).

When one compares intact nuclei versus chromatin, one might expect a decrease in specific bands which would reflect a loss of nuclear membrane material and of nucleoplasmic components. We were not able to systematically detect any differences between intact nuclei and chromatin. It is possible that the components of the nuclear membrane and the nucleoplasm were insoluble in the lysing step or may represent a pool of proteins readily exchangeable with the chromatin (Stein and Thrall, 1973). All the DNA is recovered in the pellet following lysis of the nuclei.

Although proteases are often mentioned as important components of chromatin, we do not see significant differences in chromatin prepared in the presence and absence of a protease inhibitor, TPCK. Following centrifugation of chromatin, material is immediately homogenized in the sample buffer solution containing SDS and 2-mercaptoethanol and dialyzed or frozen. The concentration of SDS and 2-mercaptoethanol should be sufficient to completely bind and denature the proteins (Weber and Osborn, 1975). Reduction with other reducing agents did not produce a difference in the banding patterns of the material on the gels.

Boiling and mixing the chromatin prior to applying it to gels did have an effect on the separation. Without such treatment, bands in the high molecular weight regions of the gels did not separate. The low pH of the histone extraction procedure was apparently sufficient to inactivate the histone proteins. No differences were seen in the histones prepared with or without TPCK.

Transcriptional Activity of Chromatin

Three separate modifications of the transcription assay procedure all showed a consistent increase in the quantitative assessment of

template capacity of chromatin from the cells grown in the presence of serum as compared with material from "differentiated" cells. This is consistent with work reported for other proliferating systems such as HeLa cells and WI38 fibroblasts (Stein and Farber, 1972; Stein et al., 1976). No attempt was made to determine if qualitative differences in transcription occurred, although one would expect to see such differences. Large amounts of material would be required to determine if differences were present between the RNA species made from chromatin templates prepared from cells grown under different culture conditions. Given the large number of proteins which have been reported to differ under the different culture conditions, we might expect to see differences reflecting the different messenger RNAs.

Bondy et al. (1974) reported that the total synthesis of cellular RNA is decreased in cells in the "differentiated" conditions (induced by phosphodiesterase inhibitors), but the percent of the RNA which contains polyadenylic acid (polyA) tails, and hence is thought to represent messenger RNA, is increased in these cells. In a later study by this same group (Prasad et al. 1975), they verified that the amount of polyA-containing cytoplasmic RNA is increased in cells induced to "differentiate" by increases in cyclic AMP and by X-ray when compared with cells grown in medium with serum. However, the amount of polyA-RNA did not change in cells treated with serum-free medium or with sodium butyrate. On the basis of comparisons of actinomycin D binding studies, they concluded that similar amounts of DNA are available for transcription in "differentiated" cells. Actinomycin D binding is not a direct measure of transcription, however. It indicates only that the proportion of guanine residues that are accessible to actinomycin D is similar in cells from serum-free cultures and from cultures in complete medium with serum.

Glazer and Schneider (1975) assessed the ability of a number of agents to induce "differentiation" and to affect RNA synthesis in an uncloned line of neuroblastoma cells. They found that RNA synthesis, both of ribosomal RNA and heterogeneous nuclear RNA, was decreased by approximately 30% in cultures of cells exposed to db cAMP when compared with cultures of cells grown with serum. They accounted for this difference by differences in the uptake of ^3H -uridine by the cells under the two growth conditions. No difference in RNA species could be detected by polyacrylamide electrophoresis of samples from cells incubated in the two media for 48 hours.

None of these studies actually analyze the RNA species which are synthesized with DNA as a template in the cells under the different growth conditions. Only an estimate of the quantitative amounts of RNA can be determined, unless one has large amounts of material to isolate individual RNA species and a specific probe, such as DNA complementary to the RNA in question, with which to carry out hybridization assays.

Chromatin Proteins

Analysis of protein spectrum. Few differences were seen on two gel systems either by visual inspection or by a quantitative analysis of the gel pattern. We must rely on the relationship between the stain intensity and the amount of protein. There is a difference in the banding patterns between chromatin from cells grown in serum and cells grown in serum-free medium in the high molecular weight regions. There is no way of determining differences within the bands with this gel system. Polypeptides are separated only by molecular weight. There may be more than one polypeptide per band which would be resolved by some other analytical method as suggested in the review by Stein et al. (1976).

No measures have been made of the enzyme activities of any of the components of the chromatin or of any other functional property of the polypeptides. They cannot be recovered from this gel system in sufficient quantity to characterize further and potential enzyme activity may be lost by the denaturation necessary to solubilize the proteins and fractionate them by molecular weight.

Histones. No changes in the migration patterns of the histones from isolated nuclei on the Panyim and Chalkley gels were seen. No separation is possible between the histones and the nonhistones which migrate together on the gels. Tryptophan incorporation, indicating the presence of nonhistones, is minor in the regions of the histones on the SDS gels.

Metabolism of Chromatin Proteins

Total proteins. We believe that nonhistone proteins are synthesized in the cytoplasm (Stein and Baserga, 1970) rather than in the nucleus as discussed by Fleischer-Lambropoulos and Reinsch (1975). They are synthesized throughout the cell cycle in continuously dividing cells and have different turnover kinetics at different times of the cell cycle for different subfractions of these polypeptides (Borun and Stein, 1972).

Both leucine and tryptophan incorporation patterns show and increase in turnover of the polypeptides which migrate in the high molecular weight regions of the SDS-polyacrylamide gels in samples of chromatin from cells grown in serum as compared with cells grown in no serum. As mentioned previously, it is difficult to interpret these types of data for the region of the gel where polypeptides of 11,000 to 22,000 molecular weight migrate due to the presence of the histone polypeptides in these regions. The differences in synthesis are consistent with qualitative

differences in the high molecular weight region at the top of the gel. The molecular weight assignment of this set of bands is tentative since it has been suggested that glycoproteins migrate anomalously in this type of gel system (Weber and Osborn, 1975). Migration of individual polypeptides depends on charge characteristics of the SDS-polypeptide complex. For at least one protein, the H1 histone, this is known to result in an anomalous migration pattern (Hayashi *et al.*, 1974). We do not know whether the increase in incorporation occurs in the same polypeptides or in the synthesis of different high molecular weight components. It would be useful to apply a second separation procedure, such as salt extraction or two-dimensional polyacrylamide gel electrophoresis, to such material.

Kelly (1975) discusses in great detail the limitations of electrophoretic fractionation procedures in studies of mouse brain proteins. He claims to have resolved 500 different proteins which were calculated to represent only 0.015 of the estimated total brain proteins. From calculations setting the lower limit of resolution of proteins by gradient gel electrophoresis on the order of 0.2% (wt/wt), he suggests that a protein must exist in tissue at the level of at least 10^{-5} grams, or 2×10^7 molecules per cell, in order to be able to resolve it on gels with Coomassie Blue staining. His paper represents an elegant study of the spectrum of proteins present in various subcellular fractions of brain tissue and a candid discussion of the limitations of methods. From his calculations and estimates, it can be readily appreciated that a protein must exist in macro quantities in order for differences to be detectable with methods currently in use.

Histone synthesis. Histones are synthesized on cytoplasmic ribosomes. The data reported suggest that histone synthesis increases in

cell cultures where there is an increase in cell number; however, the results are not consistent from one experiment to the next. There may be a variable level of cell division and histone synthesis across experiments. The specific activities of the various histone fractions cannot be directly compared across experiments since cells are grown in different batches of medium and, especially, with different lots of serum and db cAMP. The possibility of DNA synthesis in these cells being uncoupled from histone synthesis remains to be tested further.

Post-translational Modifications of Nonhistone Polypeptides

Post-translational modifications of nuclear proteins has already been discussed in terms of the possible role of such modifications on the regulation of gene expression. Acetylation does not commonly occur in the nonhistone polypeptides and, in fact, was not significant in studies of WI38 fibroblasts or in HeLa cells (M. Krause and R. Filker, personal communication). Similarly, our studies of acetylation of nuclear proteins of neuroblastoma cells revealed that acetylation occurred only of the histone polypeptides to a significant degree.

Phosphorylation of chromatin proteins has been correlated with modifications of gene activity in a number of cell lines (Kleinsmith, 1974, 1975; Stein *et al.*, 1974). Serine and threonine residues of chromatin proteins are the substrate for phosphorylation. A high turnover of the incorporated phosphate occurs. In the neuroblastoma cell chromatin, high molecular weight components of the nonhistone proteins appeared to be significantly phosphorylated, although phosphorylation of histone polypeptides also occurred to a significant degree. We found no significant differences which could be defined in the pattern of phosphorylation of the nonhistones. In fact, no direct phosphate

determination was carried out in order to determine the extent to which the nonhistones were phosphorylated. The majority of ^{32}P incorporation occurred into the high molecular weight regions of the gels. We cannot rule out the possibility of the inclusion of nucleic acids in this region of the gel (Pumo et al., 1975).

Post-translational Modifications of the Histone Polypeptides

Histones were acetylated to a significant degree following a 60-minute pulse with ^3H -acetate. No differences in acetylation were detectable among the histones isolated from nuclei of neuroblastoma cells which had been grown for 24 hours in the various media.

Phosphorylation of histones also occurred to a significant degree. Histone H1 was the most highly phosphorylated, consistent with findings in other cell types (MacGillivray and Rickwood, 1974). However, when comparisons were made of the phosphorylation of nuclear histones from cells grown under various media conditions, the differences seen were not consistent from experiment to experiment.

DNA Synthesis and Histone Synthesis

Initial studies to define the relationship between DNA synthesis and histone synthesis involved a comparison of the level of DNA synthesis, as measured by thymidine incorporation into acid insoluble material of isolated nuclei. Approximately the same levels of DNA synthesis were found in all three culture conditions. Published values of DNA/cell suggested that a decrease in DNA/cell should occur in the "differentiated" cells and thus indicate that the cells were blocked in G1. However, the constant level of DNA synthesis under all culture conditions could be explained if it was assumed that DNA replication continues in cells growing in serum-free medium, but the cells do not divide. Autoradiographic experiments or microspectrophotometric measurements and accurate

identification of each cell (with respect to its state of "differentiation") are required to answer this question.

An attempt was made to use cytosine arabinoside to investigate the relationship between histone synthesis and DNA synthesis in the mouse neuroblastoma cells. The concentration of cytosine arabinoside employed (40 $\mu\text{g/ml}$) inhibited the incorporation of thymidine into acid-insoluble material to the same degree in all three culture conditions (approximately 40 to 50%). However, no consistent differences were seen in histone synthesis analyzed as described for the non-inhibited conditions above.

The use of cytosine arabinoside in previously published studies suggested that it induced differentiation of the neuroblastoma cells itself in concentrations three orders of magnitude less than we used (Kates *et al.*, 1971). Other studies employing cytosine arabinoside did not find the same effect (Schubert and Jacob, 1970; Schubert *et al.*, 1973; Byfield and Karlsson, 1973). Slightly different culture conditions were used in each of the experiments cited above. The problem is complex and remains to be resolved. More information is needed about the transport and uptake of cytosine arabinoside by the mouse neuroblastoma cells under different growth conditions, about the effective concentration ranges for DNA synthesis inhibition and toxicity, and the time of exposure and route of metabolism.

Summary

In conclusion, we have presented an initial characterization of the genome of the mouse neuroblastoma cell line grown under three culture conditions which have been extensively studied by other laboratories and shown to result in alterations of the neuron-like properties of the cells. We have shown that the template capacity for DNA-dependent RNA

synthesis of chromatin isolated from such cells is increased several-fold when the neuroblastoma cells are grown in serum-free medium or exposed to db cAMP as compared with cells grown in medium containing serum.

This functional change in the chromatin is accompanied by a change in the synthesis or turnover of high molecular weight components of the nonhistone proteins. Other potential alterations in histone and non-histone metabolism such as acetylation and phosphorylation do not seem to occur consistently from experiment to experiment. The limits of resolution of the methods available are discussed.

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BIOGRAPHICAL SKETCH

Michelle Louise Simon was born and grew up in Los Angeles, California, where she attended high school at Hollywood High and honors courses at UCLA. Her scientific career officially began at the University of Alaska, College, Alaska, in 1963, where she participated in a Summer Honors Program sponsored by the National Science Foundation. She attended Mills College in Oakland, California, as a freshman, and then spent three years at the University of Southern California. She transferred to the University of California Irvine, and continued to work on a project begun as a participant in an undergraduate honors program sponsored by NSF. The project was supervised by Dr. James McGaugh, Department of Psychobiology, and involved a study of the effectiveness of pentylenetetrazol as an agent to enhance learning and memory in mentally retarded children. She graduated with Honors in Biology from UCI in June, 1969.

As a graduate student in the Developmental Biology Laboratory of the Department of Development and Cell Biology at UCI, Ms. Simon worked with Dr. Peter Bryant and Dr. Howard Schneiderman on problems of cellular determination and differentiation during the early development of Drosophila. She received an M.S. in Developmental Biology from UCI in December, 1973.

While at the University of Florida Ms. Simon studied in the departments of Neuroscience and Biochemistry, and worked in the laboratory of Dr. Owen M. Rennert on problems of defective myelination in mutant mice. The nuclear protein studies reported here were conducted in the laboratory

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Ms. Simon is currently a medical student at the University of Florida.

She is a student member of the Society of Neuroscience, the American Society of Cell Biology, and the American Medical Women's Association. She is also a member of the Association for Women in Science, Women in Cell Biology, and the American Medical Student Association.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



Owen M. Rennert, Chairman
Professor of Neuroscience
Pediatrics and Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate in scope and quality, as a dissertation for the degree of Doctor of Philosophy.



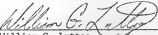
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